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(71) Applicants: ALBERT EINSTEIN COLLEGE OF MEDICINE OF YESHIVA UNIVERSITY [US/US]; 1300 Morris Park Avenue, Bronx, NY 10461 (US). UNIVERSITY OF PITTSBURGH [US/US]; 911 William Pitt Union, Pittsburgh, PA 15260 (US).

(72) Inventors: JACOBS, William, R. ; 163 Fordham Street, City Island, NY 10464 (US). HATFULL, Graham ; 5 Forbes Terrace, Pittsburgh, PA 15217 (US).

(74) Agents: OLSTEIN, Elliot, M. et al.; Carella, Byrne, Bain, Gilfillan, Cecchi & Stewart , 6 Becker Farm Road, Roseland, NJ 07068 (US).

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(54) Title: DNA CAPABLE OF SITE-SPECIFIC INTEGRATION INTO MYCOBACTERIA

(57) Abstract

A DNA for integrating DNA into a mycobacterium chromosome portion which includes a first DNA sequence which is a phage DNA portion encoding bacteriophage integration into a mycobacterium chromosome, and a second DNA sequence encoding a protein or polypeptide heterologous to the mycobacterium in which the DNA is to be integrated. Such DNA may be integrated into mycobacteria, which may then be administered as a vaccine and/or therapeutic agent.

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DNA CAPABLE OF SITE-SPECIFIC INTEGRATION INTO MYCOBACTERIA

This invention relates to DNA capable of integrating into mycobacterial chromosomes. More particularly, this invention relates to DNA which is capable of site-specific integration into a mycobacterium chromosome while containing a DNA sequence encoding a protein heterologous to the mycobacterium in which the DNA is integrated.

Certain mycobacteria represent major pathogens of man and animals. For example, tuberculosis is generally caused in humans by Mycobacterium tuberculosis, and in cattle by Mycobacterium bovis, which may also be transmitted to humans and other animals. Mycobacteria leprae is the causative agent of leprosy. M. tuberculosis and mycobacteria of the avium-intracellulare-scrofulaceum group (MAIS group) represent major opportunistic pathogens of patients with acquired immune deficiency syndrome (AIDS). M. pseudotuberculosis is a major pathogen of cattle.

On the other hand, Bacille Calmette-Guerin, or BCG, an avirulent strain of M. bovis, is widely used in human vaccines, and in particular is used as a live vaccine, which is protective against tuberculosis. BCG is the only childhood vaccine which is currently given at birth, has a very low incidence of adverse effects, and can be used repeatedly in an individual (eg., in multiple forms). In addition, BCG and other mycobacteria (eg.,

M. smegmatis), employed in vaccines, have adjuvant properties among the best currently known and, therefore, stimulate a recipient's immune system to respond to antigens with great effectiveness.

It has been suggested by Jacobs, et al, Nature, Vol. 327, No. 6122, pgs. 532-535 (June 11, 1987) that BCG could be used as a host for the construction of recombinant vaccines. In other words, it was suggested to take an existing vaccine (in this case against tuberculosis) and expand its protective repetoire through the introduction of one or more genes from other pathogens. Because BCG vaccines are administered as live bacteria, it is essential that any foreign antigens, polypeptides, or proteins expressed by the bacteria are not lost from the bacteria subsequent to vaccination.

Transformation, the process whereby naked DNA is introduced into bacterial cells, has been carried out successfully in mycobacteria. Jacobs, et al (1987), hereinabove cited, have described transformation of mycobacteria through chemical methods, and Snapper, et al. PNAS, Vol. 85, pgs. 6987-6991 (September 1988) have described transformation of mycobacteria by electroporation. Electroporation can give from 10^5 to 10^6 transformants per μg of plasmid DNA and such plasmid DNA's may carry genes for resistance to antibiotic markers such as kanamycin (Snapper, et al 1988) to allow for selection of transformed cells from non-transformed cells.

Jacobs, et al (1987) and Snapper, et al (1988) have also described the use of cloning vehicles, such as plasmids and bacteriophages, for carrying genes of interest into mycobacteria.

Combination of the above-mentioned techniques, along with standard tools of molecular cloning (e.g., use of restriction enzymes, etc.) allows the cloning of genes of interest into vectors and introduction of such genes into mycobacteria. To express these genes, it is important to have available signals for gene expression, in particular, transcription promoter

elements. Such promoter elements have been isolated from mycobacterial heat shock genes, and used to express foreign antigens in mycobacteria.

Plasmids currently available for use in mycobacteria, however, are not stably maintained, and are readily lost during non-selective growth. If recombinant mycobacteria expressing genes of interest are to be employed as vaccines against a particular antigen or as a means of providing a therapeutic agent to a host, which is expressed by the mycobacteria, it is crucial that such genes not be lost from the recombinant mycobacteria subsequent to their administration.

It is an object of the present invention to introduce into mycobacteria and ultimately express a variety of heterologous genes, including, but not limited to, genes for protective antigen(s) for a variety of pathogens, and/or for other therapeutic agents, thereby enabling one to produce effective vaccines against such pathogens and/or to provide effective therapeutic agents, whereby such genes will not be lost subsequent to vaccination or administration of the therapeutic agent.

In accordance with an aspect of the present invention, there is provided a DNA which comprises a first DNA sequence which is a phage DNA portion encoding bacteriophage integration into a mycobacterium chromosome, and a second DNA sequence encoding at least one protein or polypeptide which is heterologous to the mycobacterium in which the DNA is to be integrated.

The term "phage DNA portion", as used herein, means that the DNA sequence is derived from a phage and lacks the DNA which is required for phage replication.

Bacteriophages from which the phage DNA portion may be derived include, but are not limited to, mycobacteriophages, such as but not limited to the L5, L1, Bxb1 and TM4 mycobacteriophages; the lambda phage of E.coli; the toxin phages

of Corynebacteria; phages of Actinomycetes and Norcadia, the ø C31 phage of Streptomyces; and the P22 phage of Salmonella. Preferably, the phage DNA portion encodes mycobacteriophage integration into a mycobacterium chromosome.

In a preferred embodiment, the first DNA sequence includes DNA encoding integrase, which is a protein that provides for integration of the DNA into the mycobacterial chromosome. Most preferably, the first DNA sequence also includes DNA which encodes an AttP site.

The DNA sequence encoding the AttP site and the integrase provides for an integration event which is referred to as site-specific integration. DNA containing the AttP site and the integrase gene is capable of integration into a corresponding AttB site of a mycobacterium chromosome.

It is to be understood that the exact DNA sequence encoding the attP site may vary among different phages, and that the exact DNA sequence encoding the attB site may vary among different mycobacteria.

The integration event results in the formation of two new junction sites called AttL and AttR, each of which contain part of each of AttP and AttB. The inserted and integrated non-phage DNA which includes the first and second DNA sequences, is flanked by the AttL and AttR sites. The insertion and integration of the phage DNA portion results in the formation of a transformed mycobacterium.

Applicants have found that, when employing the phage DNA portion of the present invention for integration into mycobacterial chromosome, the gene(s) of interest which is integrated into the mycobacterial chromosome is not lost following non-selective growth of the mycobacteria. Thus, the gene(s) of interest may be expressed by the mycobacteria following such non-selective growth, thus making such tranformed mycobacteria excellent vehicles to be employed in vaccines or pharmaceuticals whereby such mycobacteria will express antigens

and/or therapeutic agents of interest subsequent to administration of the recombinant mycobacteria to a host.

Mycobacteria into which the phage DNA portion may be integrated include, but are not limited to, Mycobacterium bovis-BCG, M. smegmatis, M. avium, M. phlei, M. fortuitum, M. lufu, M. paratuberculosis, M. habana, M. scrofalaceum, M. leprae and M. intracellulare. In a preferred embodiment, the DNA is integrated into Mycobacterium bovis- BCG.

The second DNA sequence which encodes a protein heterologous to mycobacteria may be DNA which is all or a portion of a gene encoding protein(s) or polypeptide(s) of interest; DNA encoding a selectable marker or markers; or DNA encoding both a selectable marker or markers and at least one protein or polypeptide of interest.

Proteins or polypeptides of interest, which may be encoded by the second DNA sequence include, but are not limited to, antigens, anti-tumor agents, enzymes, lymphokines, pharmacologic agents, immunopotentiators, and reporter molecules of interest in a diagnostic context.

Antigens for which the second DNA sequence may encode include, but are not limited to, Mycobacterium leprae antigens; Mycobacterium tuberculosis antigens; Rickettsia antigens; malaria sporozoites and merozoites; diphtheria toxoids; tetanus toxoids; Clostridium antigens; Leishmania antigens; Salmonella antigens; Borrelia antigens; Mycobacterium africanum antigens; Mycobacterium intracellulare antigens; Mycobacterium avium antigens; Treponema antigens; Pertussis antigens; Schistosoma antigens; Filaria antigens; Herpes virus antigens; influenza and parainfluenza virus antigens; measles virus antigens; mumps virus antigens; hepatitis virus antigens; Shigella antigens; Neisseria antigens; rabies antigens, polio virus antigens; Rift Valley Fever virus antigens; dengue virus antigens; measles virus antigens; Human Immunodeficiency Virus (HIV) antigens; respiratory syncytial virus (RSV) antigens; snake venom antigens;

and Vibrio cholera antigens. Enzymes which may be encoded include, but are not limited to, steroid enzymes.

Anti-tumor agents which may be encoded by the second DNA sequence include, but are not limited to, interferon-α, interferon-β, or interferon- , and tumor necrosis factor, or TNF. Lymphokines which may be encoded include, but are not limited to, interleukins 1 through 8.

Reporter molecules which may be encoded include, but are not limited to, luciferase, B-galactosidase, B-glucuronidase, and catechol dehydrogenase.

Other peptides or proteins which may be encoded by the second DNA sequence include, but are not limited to, those which encode for stress proteins, which can be administered to evoke an immune response or to induce tolerance in an autoimmune disease (e.g., rheumatoid arthritis).

Selectable markers which may be encoded include, but are not limited to, the kanamycin resistance marker, the neomycin resistance marker, the chloroamphenicol resistance marker, and the hygromycin resistance marker.

The phage DNA portion of the present invention, which includes the first DNA sequence encoding mycobacterium phage integration into a mycobacterium chromosome, and the second DNA sequence encoding at least one protein or polypeptide heterologous to mycobacteria, may be constructed through genetic engineering techniques known to those skilled in the art. In a preferred embodiment, the phage DNA portion may be a plasmid including, in addition to the DNA encoding integration and the DNA encoding a heterologous protein, an origin of replication for any of a wide variety of organisms, which includes, but is not limited to, E.coli, Streptomyces species, Bacillus species, Staphylococcus species, Shigella species, Salmonella species and various species of pneumococci. Most preferably, the plasmid includes an origin of replication for E.coli.

The phage DNA portion also may include a suitable promoter. Suitable promoters include, but are not limited to, mycobacterial promoters such as the BCG HSP60 and HSP70 promoters; mycobactin promoters of M. tuberculosis and BCG, the superoxide dismutase promoter, the α-antigen promoter of M. tuberculosis and BCG, the MBP-70 promoter, the 45 kda antigen promoter of M. tuberculosis and BCG; and the mycobacterial asd promoter; the mycobacterial 14 kda and 12 kda antigen promoters; mycobacteriophage promoters such as the Bxb1 promoter, the L1 and L5 promoters, and the TM4 promoters; E.coli promoters; or any other suitable promoter. The selection of a suitable promoter is deemed to be within the scope of those of ordinary skill in the art from the teachings contained herein.

The promoter sequence may, in one embodiment, be part of an expression cassette which also includes a portion of the gene normally under the control of the promoter. For example, when a mycobacterial HSP60 or HSP70 promoter is employed, the expression cassette may include, in addition to the promoter, a portion of the gene for the HSP60 or HSP70 protein. When the expression cassette and the DNA encoding the heterologous protein or polypeptide are expressed, the protein expressed by the cassette and the DNA encoding the heterlogous protein or polypeptide is a fusion protein of a fragment of a mycobacterial protein (eg., the HSP60 or HSP70 protein), and the heterologous protein.

In a preferred embodiment, the transcription initiation site, the ribosomal binding site, and the start codon, which provides for the initiation of the translation of mRNA, are each of mycobacterial origin. The stop codon, which stops translation of mRNA, thereby terminating synthesis of the heterologous protein, and the transcription termination site, may be of mycobacterial origin, or of other bacterial origin, or such stop codon and transcription termination site may be those of the DNA encoding the heterologous protein or polypeptide.

The phage DNA portion may be employed, as hereinabove indicated, for integration into a mycobacterial chromosome, thereby transforming the mycobacteria, and whereby the mycobacteria will express protein(s) or polypeptide(s) heterologous to mycobacteria. Such mycobacteria may be utilized in the production of a vaccine or a therapeutic agent, depending upon the protein(s) or polypeptides expressed by the transformed mycobacteria. To form such a vaccine or therapeutic agent, the transformed mycobacteria are administered in conjunction with a suitable pharmaceutical carrier. As representative examples of suitable carriers there may be mentioned: mineral oil, alum, synthetic polymers, etc. Vehicles for vaccines and therapeutic agents are well known in the art and the selection of a suitable vehicle is deemed to be within the scope of those skilled in the art from the teachings contained herein. The selection of a suitable vehicle is also dependent upon the manner in which the vaccine or therapeutic agent is to be administered. The vaccine or therapeutic agent may be in the form of an injectable dose and may be administered intramuscularly, intravenously, orally, intradermally, or by subcutaneous administration.

Other means for administering the vaccine or therapeutic agent should be apparent to those skilled in the art from the teachings herein; accordingly, the scope of the invention is not to be limited to a particular delivery form.

When the transformed mycobacteria are employed as a vaccine, such a vaccine has important advantages over other presently available vaccines. Mycobacteria have, as hereinabove indicated, adjuvant properties among the best currently known and, therefore, stimulate a recipient's immune system to respond with great effectiveness. This aspect of the vaccine induces cell-mediated immunity and thus is especially useful in providing immunity against pathogens in cases where cell-mediated immunity appears to be critical for resistance. Also, mycobacteria may stimulate long-term memory or immunity. It thus may be possible

to prime long-lasting T cell memory, which stimulates secondary antibody responses neutralizing to the infectious agent or the toxin. Such priming of T cell memory is useful, for example, against tetanus and diphtheria toxins, pertussis, malaria, influenza virus, Herpes virus, rabies, Rift Valley fever virus, dengue virus, measles virus, Human Immunodeficiency Virus (HIV), respiratory syncytial virus, human tumors, and snake venoms. Another advantage in employing mycobacteria transformed with the phage DNA portion of the present invention as a vaccine or a therapeutic agent is that mycobacteria in general have a large genome (i.e., approximately 3×10^6 base pairs in length). Because the genome is large, it is able to accommodate a large amount of DNA from other source(s), and may possibly be employed to make a vaccine and/or therapeutic agent containing DNA sequences encoding more than one antigen and/or therapeutic agent.

Also, because the integrated gene(s) of interest has not been lost following non-selective growth of transformed mycobacteria, the gene(s) of interest will continue to be expressed by the transformed mycobacteria following administration of the mycobacteria to a host. Such mycobacteria, therefore, are effective vehicles for expressing antigens which stimulate an immune response, or for the expression of therapeutic(s) agents such as anti-tumor agents and/or anti-cancer agents.

The invention will now be described with respect to the following examples; however, the scope of the present invention is not to be limited thereby.

Example 1

A. Identification of the DNA sequences of the attachment sites, attB, attL, and attR, of M.smegmatis.

Using standard technologies, a lambda EMBL3 library was prepared from BamHI digested $mc^2 61$ chromosomal DNA ($mc^2 61$ is a

strain of M. smegmatis which includes an M. smegmatis chromosome into which has been integrated the genome of mycobacterial phage L5) and digested with Bam HI. Phage L5 contains DNA having restriction sites identical to those of phage L1 (Snapper, et al. 1988), except that L5 is able to replicate at 42°C and phage L1 is incapable of such growth. This library was then probed with a 6.7 kb DNA fragment isolated from the L5 genome that had been previously identified as carrying the attP sequence (Snapper, et al 1988). One of the positive clones was plaque purified, DNA prepared, and a 1.1 kb Sal I fragment (containing the AttL sequence) sub-cloned into sequencing vector pUC119. The DNA sequence of this fragment was determined using a shotgun approach coupled with Sanger sequencing. By isolating and sequencing the attL junction site and comparing this to the DNA sequence of L5 that was available, a region was determined where the two sequences aligned but with a specific discontinuity present. The discontinuity represents one side of a core sequence, which is identical in attP, attB, and attL. The region containing the recombinational crossover point is shown in Figure 1.

The attL DNA (1.1 kb Sal I fragment) was used as a probe to hybridize to a Southern blot of Bam HI digested $mc^2 6$ DNA, which is a strain of M. smegmatis which includes an M. smegmatis chromosome without any phage integration (Jacobs, et al, 1987, hereinabove cited.). A single band of approximately 6.4 kb was detected corresponding to the attB sequence of M. smegmatis. This same attL probe was used to screen a cosmid library of $mc^2 6$ (provided by Dr. Bill Jacobs of the Albert Einstein College of Medicine of Yeshiva University), and a number of positive cosmid clones were identified. DNA was prepared from these clones, and a 1.9 kb Sal I fragment (containing the attB site) that hybridizes to the attL probe was subcloned into pUC119 for sequencing and further analysis. The DNA sequence containing the core sequence was determined and is shown in Figure 1. The core

sequence, which is identical in attP, attB and attL, has a length of 43bp.

The $mc^2$61 lambda EMBL3 library was then probed with the 1.9kb SalI fragment containing the attB site. Positive plaques were identified, DNA was prepared, and analyzed by restriction analysis and Southern blots. Lambda clones were identified that contained a 3.2kb Bam HI fragment containing the putative attR site. The 3.2kb Bam HI fragment was purified and cloned into pUC119 for sequencing and further analysis.

B. Determination of attP-integrase region of L5 genome.

Concurrent with the above procedures, a significant portion of the DNA sequence of L5 had been determined and represented in several "contigs" or islands of DNA sequence. Sequences of the 6.7kb Bam HI fragment hereinabove described were determined by (a) analysis of the location of Bam HI sites in the contigs of the DNA of L5, and (b) by determining a short stretch of DNA sequence from around the Bam HI sites of plasmid pJR-1 (Figure 6), which carries the 6.7kb Bam HI fragment of L5.

A segment of DNA sequence was located that represented the 6.7kb Bam HI fragment of phage L5. Studies of other phages have shown that the integrase genes are often located close to the attP site. It was thus determined that the L5 integrase (int) gene should lie either within the 6.7kb Bam HI fragment or in a DNA sequence on either side of it. The DNA sequence in the regions was then analyzed by translating it into all six possible reading frames and searching these amino acid sequences for similarity to the family of integrase related proteins, and through computer-assisted analysis of the DNA sequence. As shown in Figure 2, there are shown two domains of reasonably good conservation among L5 integrase and other integrases, and three amino acid residues that are absolutely conserved in domain 2. (See Yagil, et al., J. Mol. Biol., Vol. 207, pgs. 695-717 (1989), and Poyart-Salmeron, et al., J. EMBO, Vol. 8, pgs. 2425-2433 (1989)). A region was identified, and analysis of the

corresponding DNA sequence showed a reading frame that could encode for a protein of approximately 333 amino acids. These observations identified the putative int gene.

The location of the int gene was not within the 6.7kb Bam HI fragment; however, it was very close to it with one of the Bam HI sites (that defines the 6.7kb Bam HI fragment) less than 100 bp upstream of the start of the gene. Analysis of the Bam HI sites showed that the int gene lay within a 1.9kb Bam HI fragment located adjacent to the 6.7kb Bam HI fragment. This 1.9kb Bam HI fragment was cloned by purification of the fragment from a Bam HI digest of L5 DNA and cloning into pUC 119, to generate pMH1 (Figure 7).

From a combination of the above approaches, a schematic of the organization of the attP-int region of L5 was constructed (Figure 3), and the gene sequence of the attP-int region is given in Figure 4.

C. Construction of pMH5.

The 6.7kb Bam HI fragment of mycobacteriophage L5, which contains the attP site, as hereinabove described, was cloned into the Bam HI site of pUC 119 (Figure 5). This was achieved by purifying the 6.7kb Bam HI fragment from a Bam HI digest of L5 DNA separated by agarose gel electrophoresis and ligating with Bam HI cut pUC 119. DNA was prepared from candidate recombinants and characterized by restriction enzyme analysis and gel electrophoresis. A recombinant was identified that contained the 6.7kb Bam HI fragment of L5 cloned into pUC 119. This plasmid was named pJR-1, as shown in Figure 6.

Analysis of DNA sequence data from a project to sequence L5 showed that a 1.9kb Bam HI fragment adjacent to the 6.7kb Bam HI fragment hereinabove described contained the integrase gene.

A plasmid containing a 1.9kb Bam HI fragment containing the DNA encoding for the integrase cloned into the Bam HI site of pUC 119 was constructed. The 1.9kb fragment was purified from a Bam

HI digest of L5 DNA and cloned into the Bam HI site of pUC 119. Construction of the recombinant was determined by restriction analysis and gel electrophoresis. This plasmid was called pMH1, the construction of which is shown schematically in Figure 7.

pJR-1 was then modified by digestion with EcoRI and SnaBI (both are unique cloning sites), between which is a Bam HI site. The Eco RI-Sna BI fragment, including the Bam HI site was excised, and the plasmid was religated to form plasmid of pMH2, which contains one Bam HI site compared to two Bam HI sites contained in pJR-1. A schematic of the construction of pMH2 is shown in Figure 8.

The 1.9kb Bam HI fragment, which includes the integrase gene, was purified from a Bam HI digest of pMH1 and ligated to Bam HI digested pMH2. Recombinants were identified as above and the orientation of the 1.9kb fragment determined. A plasmid called pMH4 was thus constructed (Figure 9) in which the region from the Sna BI site (upstream of attP) through to the Bam HI site (downstream of the integrase gene) was identical to that in L5.

pMH4 was digested with HindIII (unique site) and was ligated to a 1kb HindIII fragment purified from pKD43 (supplied by Keith Darbyshire of the Nigel Gindley Laboratory) that contains the gene determining resistance to kanamycin. Recombinants were identified and characterized as above. This plasmid is called pMH5. A schematic of the construction of pMH5 is shown in Figure 10.

D. Integration of pMH5 into attB of M. smegmatis.

Plasmids pYUB12 (a gift from Dr. Bill Jacobs a schematic of the formation of which is shown in Figure 20), pMD01 (Figure 11), and pMH5 were electroporated, with four different concentrations of plasmid DNA over a 1,000-fold range, into M. smegmatis strain mc^2155, a strain which is able to support plasmid replication. In Examples 1 and 2, all electroporation procedures of M. smegmatis, or of BCG, were carried out as follows:

Cultures of organism were grown in Middlebrook 7H9 media, as described by Snapper, et al. (1988), harvested by centrifugation, washed three times with cold 10% glycerol, and resuspended at approximately a 100 x concentration of cells.

1 µl of DNA was added to 100 µl of cells in an ice-cold cuvette and pulsed in a Bio-Rad Gene Pulser, and given a single pulse at 1.25 kv at 25 µF. 1 ml of broth was added the cells incubated for 1 hr. at 37°C for expression of the antibiotic-resistant marker. Cells were then concentrated and plated out on Middlebrook or tryptic soy media containing 15 µg/ml kanamycin. Colonies were observed after 3 to 5 days incubation at 37°C.

Each of pYUB12, pMD01, and pMH5 carries kanamycin resistance. Plasmid pYUB12 carries an origin of DNA replication, while pMD01 lacks a mycobacterial origin of replication. Plasmid pMH5 does not carry a mycobacterial origin of replication, but carries a 2kb region of phage L5 which contains the attP site and the integrase gene (Figure 4). The number of transformants were linear with DNA concentration. Plasmid pYUB12 gives a large number of transformants (2×10^5 per µg DNA) in mc^2155, while pMH5 gives 6×10^4 transformants per µg DNA, and pMD01 gives no transformants.

The above experiment was then repeated by electroporating the plasmids pYUB12, pMD01, and pMH5 into M. smegmatis strain mc^26, which does not support plasmid replication. No transformants in mc^26 were obtained from pYUB12 or pMD01, while pMH5 gave approximately 10^4 kanamycin resistant transformants in mc^26 per µg of DNA, thus indicating integration of pMH5 into the mc^26 chromosome.

DNA from six independent pMH5 transformants (four in mc^2 155 and two in mc^26) was prepared. These DNA's (along with DNA from both mc^2155 itself, and mc^2155 carrying the plasmid pYUB12) were digested with a restriction enzyme, and analyzed by Southern blot and hybridization with the M. smegmatis 1.9kb attB probe

hereinabove described. As shown in Figure 12, all six transformants have integrated into the attB site, resulting in the production of two new DNA fragments with different mobilities. If pMH5 did not integrate into the attB site, it would be expected that a single band, corresponding to the attB site in the mc^2155 control, would be obtained.

E. Construction of pMH9.2 and pMH9.4

pUC119 was digested with HindIII, and a 1kb HindIII fragment, containing a kanamycin resistance gene, purified from pKD43, was ligated to the HindIII digested pUC119 to form pMH8 (Figure 13.). A 2kb SalI fragment of pMH5 (bp 3226-5310), which carries the attP and integrase gene from SalI digested pMH5, was purified and inserted in both orientations relative to the vector backbone of SalI digested pMH8 to form plasmids pMH9.2 and pMH9.4 (Figures 14 and 15).

F. Stabilty of plasmids in M. smegmatis

M. smegmatis strain mc^2155 cells carrying, as a result of electroporation, plasmid pYUB12, pMH9.2 or pMH9.4, or strain mc^26 cells carrying plasmid pMH5, as a result of electroporation as hereinabove described, were grown to saturation in broth with kananmycin. Cultures were then diluted 1:100 into broth without kanamycin and grown to saturation. Two further cycles of dilution and growth were done, corresponding to about 20 generations of bacterial growth. Cultures were plated out to single colonies on non-selective plates, and approximately 100 of these colonies were patch plated onto both non-selective and selective plates. The % of colonies that were sensitive to kanamycin, thus corresponding to the percentage of cells which lost the plasmid, is given below in Table I.

Table I

	% loss
pYUB12 (mc^2155)	35
pMH5 (mc^26)	17
pMH9.2 (mc^2155)	3

pMH9.4 (mc^2155) 0

Example 2

The 1.9 kb Sal I fragment, which includes the M. smegmatis attB site as hereinabove described was cloned into pUC119, and the plasmid generated was named pMH-12. (Figure 16).

Gel purified Sal I 1.9kb M. smegmatis fragment containing attB (isolated from pMH-12) was used to probe a Southern transfer of Bam HI digested mycobacterial DNA's, including BCG substrain Pasteur, shown in Figure 17. This demonstrated that there is one Bam HI fragment of BCG that strongly hybridizes to the M. smegmatis attB probe and three hybridize weakly. The strongest hybridizing band is the fastest moving band (approximately 1.9 kb).

The same probe as above was used to probe a BCG cosmid library (provided by Dr. Bill Jacobs) and positive clones were identified. DNA was prepared from several positive clones and analyzed by restriction analysis and Southern blotting. The 1.9 kb Bam HI fragment (corresponding to the strongly hybridizing band in the Southern blot) was identified, gel purified from the cosmid DNA and cloned into pUC119. The resulting plasmid was named pMH-15. (Figure 18).

Plasmids pMH-5 and pMH9.4 were electroporated into BCG Pasteur. It was observed that pMH9.4 transforms BCG with high efficiency (approximately 10^4 transformants/μg DNA), while pMH-5 transforms BCG at low efficiency (1-10 transformants/μg DNA). DNA was prepared from BCG transformants and analyzed by Bam HI restriction and Southern blot analysis, probing with gel purified 1.9kb Bam HI BCG attB fragment from pMH-15. These data are shown in Figure 19 and show that integration of both pMH 5 and pMH 9.4 is specific to the BCG attB site (ie. the strongly cross-hybridizing fragment in BCG). This is illustrated by the loss of the 1.9kb Bam HI fragment from the transformants and the appearance of two new bands representing attL and attR junction fragments. Figure 19 shows just one of the pMH 5/BCG

transformants, although all of the four that were analyzed show that one of the bands (the largest) is smaller than expected (and different in each of the transformants), indicating that the transformation effiency of pMH 5 is low in BCG. In contrast, the four pMH 9.4 transformants are identical to each other (Figure 19) and give attR and attL junction fragments of the predicted sizes.

Example 3

A. Construction of plasmids including mycobacterial promoter expression cassette.

1. Construction of pYUB125

Plasmid pAL5000, a plasmid which contains an origin of replication of M. fortuitum, and described in Labidi, et al., FEMS Microbiol. Lett., Vol. 30, pgs. 221-225 (1985) and in Gene, Vol. 71, pgs. 315-321 (1988), is subjected to a partial Sau 3A digest, and 5kb fragments are gel purified. A 5kb fragment is then ligated to Bam HI digested pIJ666 (an. E. coli vector containing an E. coli origin of replication and also carries neomycin-kanamycin resistance, as described in Kieser, et al., Gene, Vol. 65, pgs. 83-91 (1988) to form plasmid pYUB12. A schematic of the formation of plasmid pYUB12 is shown in Figure 20. pYUB12 and pIJ666 were then transformed into M. smegmatis and BCG. Neomycin-resistant transformants that were only obtained by pYUB12 transformation confirmed that pAL5000 conferred autonomous replication to pIJ666 in M. smegmatis and BCG.

Shotgun mutagenesis by Snapper, et al (1988, hereinabove cited) indicated that no more than half of the pAL5000 plasmid was necessary to support plasmid replication in BCG. This segment presumably carried open reading frames ORF1 and ORF2, identified by Rauzier, et al., Gene, Vol. 71, pgs. 315-321 (9188), and also presumably carried a mycobacterial origin of replication. pYUB12 is then digested with HpaI and EcoRV, a 2586 bp carrying this region or segment of pAL5000 is removed and

ligated to PvuII digested pYUB8. Plasmid pYUB8 (a pBR322 derivative) includes an E. coli replicon and a kan^R (aph) gene. Ligation of the 2586 bp pYUB12 fragment to PvuII digested pYUB8 results in the formation of pYUB53, as depicted in Figure 21. Transformation of pYUB53 confirmed that the EcoRV-HpaI fragment, designated M.rep, was capable of supporting autonomous replication in BCG.

Plasmid pYUB53 was then digested with AatI, EcoRV, and PstI in order to remove the following restriction sites:

AatI 5707
EcoRI 5783
BamHI 5791
SalI 5797
PstI 5803
PstI 7252
SalI 7258
BamHI 7264
EcoRI 7273
ClaI 7298
HindIII 7304; and
EcoRV 7460

Fragment ends are then flushed with T4 DNA polymerase and religated to form plasmid pYUB125, construction of which is shown in Figure 22.

2. Elimination of superfluous vector DNA from pYUB125

792 bases of the tet gene, whch had been inactivated by prior manipulations, was eliminated by a complete NarI digest, gel purification of the 6407 bp fragment, and ligation/recirculation, transformation of E. coli strain HB101, and selection of Kan^R transformants. The construction of the resulting plasmid, pMV101, is schematically indicated in Figure 23, and the DNA sequence of pMV101, which includes markings of regions which will be deleted and of mutations, as hereinafter described, is shown in Figure 24.

3. Elimination of undesirable restriction sites in aph (kan^R) gene.

To facilitate future manipulations, the HindIII and ClaI restriction sites in the aph gene were mutagenized simultaneously by polymerase chain reaction PCR mutagenesis according to the procedure described in Gene, Vol. 77 pgs. 57-59 (1989). The bases changed in the aph gene were at the third position of codons (wobble bases) within each restriction site and the base substitutions made were designed not to change the amino acid sequence of the encoded protein.

Separate PCR reactions of plasmid pMV101 with primers ClaMut-Kan + HindRMut-Kan and HindFMut-Kan + Bam-Kan were performed at 94°C (1 min.), 50°C (1 min.), and 72°C (1 min.) for 25 cycles. The PCR primers had the following base sequences:

ClaMut-Kan

CTT GTA TGG GAA GCC CC

HindRMut-Kan

GTG AGA ATG GCA AAA GAT TAT GCA TTT CTT TCC AG

HindFMat-Kan

GTC TGG AAA GAA ATG CAT AAT CTT TTG CCA TTC TCA CCG G

Bam-Kan

CGT AGA GGA TCC ACA GGA CG

The resulting PCR products were gel purified and mixed and a single PCR reaction without primers was performed at 94°C (1 min.), 72°C (1 min.) for 10 cycles. Primers ClaMut-Kan and Bam-Kan were added and PCR was resumed at 94°C (1 min.), 50°C (1 min.), and 72°C (2 min.) for 20 cycles. The resulting PCR product (Kan. mut) was digested with BamHI and gel purified. Plasmid pMV101 was digested with ClaI and cohesive ends were filled in by Klenow + dCTP + dGTP. Klenow was heat inactivated and the digest was further digested with BamHI. The 5232 base pair fragment was gel purified and mixed with fragment Kan.mut and ligated. The ligation was transformed into E.coli strain HB101 and Kan^R colonies were screened for plasmids resistant to

ClaI and HindIII digestion. Such plasmids were designated as pMV110, which is depicted in Figure 23.

4. Elimination of sequences not necessary for plasmid replication in mycobacteria.

Plasmid pMV110 was resected in separate constructions to yield plasmids pMV111 and pMV112. In one construction, pMV110 was digested with NarI and BalI, the ends were filled in, and a 5296 base pair fragment was ligated and recircularized to form pMV111. In another construct, pMV110 was digested with NdeI and SplI, the ends were filled in, and a 5763 base pair fragment was ligated and recircularized to form pMV112. Schematics of the constructions of pMV111 and pMV112 are shown in Figure 25. These constructions further eliminated superfluous E. coli vector sequences derived from pAL5000 not necessary for mycobacterial replication. Cloning was performed in E. coli. Plasmids pMV111 and pMV112 were tested for the ability to replicate in M. smegmatis. Because both plasmids replicated in M. smegmatis the deletions of each plasmid were combined to construct pMV113.

To construct pMV113 (Figure 25), pMV111 was digested with BamHI and EcoRI, and a 1071 bp fragment was isolated. pMV112 was digested with BamHI and EcoRI, and a 3570 bp fragment was isolated, and then ligated to the 1071 bp fragment obtained from pMV111 to form pMV113. These constructions thus defined the region of pAL5000 necessary for autonomous replication in mycobacteria as no larger than 1910 base pairs.

5. Mutagenesis of restriction sites in mycobacterial replicon.

To facilitate further manipulations of the mycobacterial replicon, PCR mutagenesis was performed as above to eliminate the Sal I, EcoRI, and BglII sites located in the open reading frame known as ORF1 of pAL5000. PCR mutagenesis was performed at wobble bases within each restriction site and the base substitutions were designed not to change the amino acid sequence of the putative encoded ORF1 protein. The restriction sites were eliminated one at a time for testing in mycobacteria. It was

possible to eliminate the SalI and EcoRI without altering replication in M. smegmatis. In one construction, PCR mutagenesis was performed at EcoRI1071 of pMV113 with primers Eco Mut - M.rep and Bam-M.rep. to form pMV117, which lacks the EcoRI1071 site. Primer Eco Mut - M.rep has the following sequence:

TCC GTG CAA CGA CGT GTG TCC CGG A;

and Bam-M.rep has the following sequence:

CAC CCG TCC TGT GGA TCC TCT AC.

In another construction, PCR mutagenesis was performed at the SalI 1389 site with primer Sal Mut - M.rep and Bam-M.rep to form pMV119, which lacks the SalI 1389 site. Primer Sal Mut - M.rep has the following sequence:

TGG CGA CCG CAG TTA CTC AGG CCT.

pMV117 was then digested with ApaLI and BglII, and a 3360 bp fragment was isolated. pMV119 was digested with ApaLI and BglII, and a 1281 bp fragment was isolated and ligated to the 3360 bp fragment isolated from pMV117 to form pMV123. A schematic of the constructions of plasmids pMV117, pMV119, and pMV123 is shown in Figure 26. Elimination of the BglII site, however, either by PCR mutagenesis or Klenow fill in, eliminated plasmid replication in mycobacteria, thus suggesting that the BglII site is in proximity to, or within a sequence necessary for mycobacteria plasmid replication.

6. Construction of pMV200 series vectors.

To facilitate manipulations of all the components necessary for plasmid replication in E. coli and mycobacteria, (E. rep. and M. rep.) and selection of recombinants (Kan^R), cassettes of each component were constructed for simplified assembly in future vectors and to include a multiple cloning site (MCS) containing unique restriction sites and transcription and translation terminators. The cassettes were constructed to allow directional cloning and assembly into a plasmid where all transcription is unidirectional.

Kan^R Cassette

A DNA cassette containing the aph (Kan^R) gene was constructed by PCR with primers Kan5' and Kan$^{3'}$. An SpeI site was added to the 5' end of PCR primer Kan3', resulting in the formation of a PCR primer having the following sequence:

CTC GAC TAG TGA GGT CTG CCT CGT GAA G.

Bam HI + NheI sites were added to the 5' end of PCR primer Kan5', resulting in the formation of a PCR primer having the following sequence:

CAG AGG ATC CTT AGC TAG CCA CCT GAC GTC GGG G.

PCR was performed at bases 3375 and 4585 of pMV123, and BamHI and NheI sites were added at base 3159, and an SpeI site was added at base 4585. Digestion with BamHI and SpeI, followed by purification resulted in a 1228/2443 Kan^R cassette bounded by BamHI and SpeI cohesive ends with the direction of transcription for the aph gene proceeding from BamHI to Spe I.

E. rep. cassette

A DNA cassette containing the ColEI replicon of pUC19 was constructed by PCR with primers E.rep/Spe and E.rep/Mlu. An SpeI site was added to the 5' end of PCR primer E.rep/Spe, and an MluI site was added to the 5' end of PCR primer E.rep./Mlu. The resulting primers had the following base sequences:

E.rep./Spe

CCA CTA GTT CCA CTG AGC GTC AGA CCC

E.rep./Mlu

GAC AAC GCG TTG CGC TCG GTC GTT CGG CTG.

PCR was performed at bases 713 and 1500 of pUC19, and an MluI site was added to base 713, and a SpeI site was added to base 1500. Digestion with MluI and SpeI, followed by purification resulted in an E.rep. cassette bounded by SpeI and MluI cohesive ends with the direction of transcription for RNA I and RNA II replication primers proceeding from SpeI to MluI.

M.rep. cassette

A DNA cassette containing sequences necessary for plasmid replication in mycobacteria was constructed by PCR of pMV123 with primers M.rep/Mlu and M.rep/Bam. An MluI site was added to the 5' end of PCR primer M.rep/Mlu. A BamHI site was added to the 5' end of PCR primer M.rep/Bam. The resulting PCR primers had the following base sequences:

M.rep./Mlu

CCA TAC GCG TGA GCC CAC CAG CTC CG

M.rep./Bam

CAC CCG TCC TGT GGA TCC TCT AC

PCR was performed at bases 134 and 2082 of pMV123. An MluI site was added to base 2082. Digestion with BamHI and MluI, followed by gel purification resulted in a 1935 base pair DNA cassette bounded by MluI and BamHI cohesive ends with the direction of transcription for the pAL5000 ORF1 and ORF2 genes proceeding from MluI to Bam HI.

The Kan^R, E.rep, and M.rep PCR cassettes were then mixed in equimolar concentrations and ligated, and then transformed in E.coli strain HB101 for selection of Kan^R transformants. Colonies were screened for the presence of plasmids carrying all three cassettes after digestion with BamHI + MluI + SpeI and designated pMV200. An additional restriction site, NcoI, was eliminated from the M.rep cassette by digestion of pMV200 with NcoI, fill in with Klenow, and ligation and recircularization, resulting in the formation of pMV201. A schematic of the formation of pMV200 from pMV123 and pUC19, and of pMV201 from pMV200, is shown in Figure 27. Plasmids pMV200 and pMV201 were transformed into M. smegmatis and BCG. Both plasmids yielded Kan^R transformations, thus indicating their ability to replicate in mycobacteria.

A synthetic multiple cloning sequence (MCS) (Figure 28) was then designed and synthesized to facilitate versatile molecular cloning and manipulations for foreign gene expressions in mycobacteria, and for integration into the mycobacterial

chromosome. The synthetic MCS, shown in Figure 28, contains 16 restriction sites unique to pMV201 and includes a region carrying translation stop codons in each of three reading frames, and a transcription terminator derived from E. coli 5S ribosomal RNA (T1).

To insert the MCS cassette, pMV201 was digested with NarI and NheI, and the resulting fragment was gel purified. The MCS was digested with HinPI and NheI and, the resulting fragment was gel purified. The two fragments were then ligated to yield pMV204. A schematic of the construction of pMV204 is shown in Figure 29.

Plasmid pMV204 was then further manipulated to facilitate removal of the M.rep cassette in further constructions. pMV204 was digested with MluI, and an MluI - Not I linker was inserted into the MluI site between the M.rep and the E.rep to generate pMV206. A schematic of the construction of pMV206 from pMV204 is shown in Figure 30, and the DNA sequence of pMV206 is given in Figure 31.

7. Construction of expression cassette based on BCG HSP60.

Among the most abundant proteins in mycobacteria is the HSP60 heat shock protein (also known as the 65 kda antigen). Because abundance of the HSP60 protein in mycobacteria indicates strong HSP60 gene expression, the sequence controlling HSP60 expression was chosen to control expression of heterologous genes encoding antigens or other proteins in BCG.

The published sequence of the BCG HSP60 gene (Thole, et al, Infect. and Immun., Vol. 55, pgs. 1466-1475 (June 1987)), and surrounding sequence permitted the construction of a cassette carrying expression control sequences (i.e., promoter and translation initiation sequences) by PCR. The BCG HSP61 cassette (Figure 32) contains 375 bases 5' to the BCG HSP60 start codon, and 15 bases (5 codons) 3' to the start codon. PCR oligonucleotide primers were then synthesized. Primer Xba-HSP60, of the following sequence:

CAG ATC TAG ACG GTG ACC ACA ACG CGC C

was synthesized for the 5' end of the cassette, and primer Bam-HSP61, of the following sequence:

CTA GGG ATC CGC AAT TGT CTT GGC CAT TG

was synthesized for the 3' end of the cassette. The primers were used to amplify the cassette by PCR from BCG substrain Pasteur chromosomal DNA. The addition of the Bam HI site at the 3' end of the cassette adds one codon (Asp) to the first six codons of the HSP60 gene.

Each of pMV206 and the PCR cassette HSP61 was digested with Xba I and Bam HI. The PCR cassette was then inserted between the Xba I and Bam HI sites of pMV206, and then ligated to form pMV261, which is shown in Figure 33.

The E. coli lac Z gene was used as a reporter, or marker gene to assay the ability of the HSP61 cassette tc express heterologous genes in BCG. A BamHI restriction fragment carrying the lac Z gene was cloned into the Bam HI site of Bam HI digested pMV261, resulting in the formation of pMV261/LZ. A schematic of the construction of pMV261/LZ is shown in Figure 34. The formation of pMV261/LZ results in a fusion between the HSP60 and lac Z genes at the sixth codon of the HSP60 gene and the sixth codon of the lac Z gene. pMV261/LZ was then transformed into E. coli. Blue E. coli colonies were selected on x-gal plates for the presence of pMV261/LZ, thus indicating that the HSP60 promoter and translation initiation sequences were also active in E. coli.

pMV261/LZ was then transformed into BCG and plated on Dubos Oleic Agar plates containing x-gal. All BCG colonies resulting from this transformation exhibited blue color, thus indicating that the lac Z gene product (B-galactosidase) was expressed in BCG. SDS polyacrylamide gel electrophoresis was performed on lysates of the pMV261/LZ BCG recombinants, revealing that B-galactosidase protein was expressed to levels in excess of 10% of total BCG protein (as determined by staining with Coomassie

brilliant blue). These data indicated that BCG HSP61 expression cassette was functional in expression vector pMV261, and that substantial expression of a heterologous gene could be achieved using HSP60 - controlled expression in BCG.

Plasmid pMV261/LZ was then shown to replicate autonomously, and express the E. coli B-galactosidase, or lacZ gene, driven by the BCG promoter HSP60, in M. smegmatis and BCG.

B. Transfer of mycobacterial phage L5 integration sequences to BCG expression vector.

Plasmid pMH9.4, which includes the mycobacterial phage L5 attP site and the L5 integrase gene, was digested to completion with either KpnI + PvuII or XbaI + PvuII, and a restriction fragment of 1862 or 1847 base pairs, respectively, each of which contain the attP site and the integrase gene, were purified by agarose gel electrophoresis. Plasmid pMV261/LZ was digested with XbaI + DraI to generate either a 7569 bp or 7574 bp vector fragment. The 7569 bp fragment was ligated to the 1862 bp fragment derived from pMH9.4 to form pMV460F/LZ. The 7574 bp fragment was ligated to the 1847 bp fragment derived from pMH9.4 to form pMV460 R/LZ. Plasmids pMV460 F/LZ and pMV460R/LZ each include a mycobacterial replicon, the L5 attP site, and the L5 integrase gene. A schematic of the formation of plasmids pMV460 F/LZ and pMV460R/LZ is shown in Figure 35. To generate derivatives without the mycobacterial plasmid replicon, plasmids pMV460F/LZ and pMV460R/LZ were digested with NotI and recircularized by ligation to generate pMV360F/LZ and pMV360R/LZ. A schematic of the construction of pMV360F/LZ and pMV360R/LZ is shown in Figure 36.

Plasmids pMH9.4, pMV261/LZ, pMV460F/LZ, pMV460R/LZ, pMV360F/LZ, and pMV360R/LZ were then transformed into M. smegmatis and BCG to test their ability to replicate autonomously or integrate into the M. smegmatis or BCG chromosome. Transformation with pMH9.4, pMV261/LZ, pMV360F/LZ, and pMV360R/LZ yielded kananmycin resistant transformants of M. smegmatis and

BCG. Transformants of pMV261LZ, pMV360F/LZ, and pMV360R/LZ were shown to express E. coli B-galactosidase by SDS-polyacrylamide gel electrophoresis and X-gal assay as hereinabove described for pMV261/LZ. Plasmids pMV461F/LZ and pMV461R/LZ failed to yield kanamycin resistant transformants, thus indicating that chromosomal integration of a plasmid carrying sequences mediating autonomous replication is lethal to mycobacteria.

Example 4

A. Construction of expression cassettes based on BCG HSP70.

A partial sequence of the 5' region of the BCG HSP70 gene (which encodes for the BCG HSP70 heat shock protein, also known as the 70 kda antigen) obtained by Dr. Rick Young (MIT) permitted the construction of cassettes carrying expression control sequences (i.e., promoters and translation initiation sequences) by PCR, according to the procedures hereinabove cited. The BCG-HSP71 cassette (Figure 32) contains 150 bases 5' to the BCG-HSP70 start codon and 15 bases (5 codons) 3' to the start codon. Primer Xba-HSP70 was synthesized for the 5' end of the cassette, and primer Bam-HSP71 was synthesized for the 3' end of the cassette. The primers had the following base sequences:

Xba-HSP70

GGC CTC TAG ACC CGC ACG ACC AGC GTT AGC

Bam-HSP71

GCT AGG ATC CCC GAC CGC ACG AGC CAT GGT

The primers were used to amplify the cassette from BCG substrain Pasteur chromosomal DNA. The addition of the Bam HI site at the 3' end of the cassette adds 1 codon (Asp) to the 3' end of the HSP71 expression cassette.

Each of pMV206 and the PCR cassette HSP71 was digested with XbaI and BamHI. The PCR cassette was then inserted between the XbaI and BamHI sites of PMV 206, and then ligated to form pMV271, which is shown in Figure 33.

B. Cloning of HIV-1gag.

A BamHI-ClaI PCR cassette of HIV-1 gag was cloned between the Bam HI and Cla I sites of pMV261 and pMV271 to obtain pMV261/gag and pMV271/gag. Expression of the gag antigens in BCG was verified by the appearance of immunoreactive protein bands in Western blot analysis of BCG pMV271/gag recombinant lysates. BCG transformants, however, were never obtained with pMV261/gag, thus indicating that gag as expressed from pMV26/gag was lethal.

C. Integration of HSP60-gag expression cassette into BCG.

In order to test whether integration of an HSP-60-gag expression cassette into BCG would result is non-lethal expression of gag in BCG, it was decided that the HSP60-gag expression cassette be cloned into a plasmid (pMV307) which includes the mycobacteriophange L5 attP and integrase sequences, the construction of which is explained hereinbelow.

1. Construction of pMV307.

Plasmid pMV206 was digested with NotI to remove the mycobacterial replicon. The resulting 2209 bp fragment, which includes the aph (Kan^R) gene, the E.coli replicon and the multiple cloning site, was ligated and recircularized to form pMV205, the construction of which is schematically depicted in Figure 30.

PCR with primers XbaI-Att/Int and NheI-Att/Int was then performed on a Sal I fragment from pMH9.4, which contains the attP site and the L5 integrase gene. The resulting cassette was then digested with XbaI and NheI, and a 1789 bp fragment was gel purified. pMV205 was then digested with NheI, and the resulting fragment was ligated to the 1989 bp fragment obtained from pMH9.4 to form pMV307. A schematic of the construction of pMV307 is shown in Figure 37.

2. Construction and transformation of pMV361/gag.

The XbaI-ClaI HSP-antigen cassette, which includes the HSP60 promoter and HIV-1gag sequences, was cloned between the NotI and ClaI sites of pMV307 to form plasmid pMV361/gag. pMV361/gag was

then transformed into BCG and shown to express HIV-1gag protein by Western blot analysis with HIV-1 infected human sera.

Example 5

A series of antigen gene fragments, or cassettes, were constructed by PCR, with the exception of the gene fragment containing the gene for human tumor antigen p97, as indicated in Table 2, and cloned into various restriction sites of pMV261 and pMV271 to form new constructs of the pMV261 and pMV271 series. The antigen genes, antigen gene fragments, cloning sites used in pMV261 and pMV271 and the names of the resulting constructs, are given below in Table 2.

The following constructs:

pMV261/P97
pMV271/P97
pMV261/CS
pMV271/CS
pMV261/SM97
pMV271/SM97
pMV271/HIV-1-gag
pMV261/HIV-1gp120
pMV271/HIV-1gp120
pMV261/HIV-1-gp41
pMV271/HIV-1-gp41
pMV261/HIV2gag
pMV271/HIV2gag
pMV261/HIV2-gp120
pMV271/HIV2-gp120
pMV261/HIV2-gp41; and
pMV271/HIV2-gp41.

were transformed into BCG, and the presence of the corresponding antigens in BCG was verified by the appearance of immunoreactive protein bands in Western blot analysis of BCG recombinant lysates.

Example 6

Antigen gene expression cassettes, which include a promoter sequence and a heterologous gene sequence, were excised from the pMV261 and pMV271 derivatives with NotI and a second restriction enzyme site (Pvu II, Eco RI, Sal I, Cla I or Hind III) and cloned into the integrating plasmid pMV307 between the NotI site and a second enzyyme site (Pvu II, Eco RI, Sal I, Cla I or Hind III) to form the pMV 361/XXX and pMV371/XXX series of plasmids (e.g., pMV361/HIV-Igp120). The backbones of these series of plasmids (pMV361 and pMV371) are shown in Figure 38.

The following plasmids:

pMV361/P97

pMV371/P97
pMV361/CS
pMV371/CS
pMV361/SM97
pMV371/SM97
pMV361/HIV-1gag
pMV371/HIV-1gag
pMV361/HIV-1gp120
pMV371/HIV-1gp120
pMV361/HIV-1gp41
pMV371/HIV-1gp41
pMV361/HIV2gag
pMV371/HIV2gag
pMV361/HIV2-gp120; and
pMV361/HIV2-gp41.

were transformed into BCG and shown to express the corresponding antigens by Western blot analysis with the appropriate antigen-specific human sera.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

WHAT IS CLAIMED IS:

1. A DNA for integrating DNA into a mycobacterium chromosome, comprising:

a first DNA sequence which is a phage DNA portion encoding bacteriophage integration into a mycobacterium chromosome; and

a second DNA sequence encoding a protein or polypeptide which is heterologous to the mycobacterium in which the DNA is to be integrated.

2. The DNA of Claim 1 wherein said phage DNA portion encodes mycobacteriophage integration into a mycobacterium chromosome.

3. The DNA of Claim 1 wherein said first DNA sequence includes DNA encoding integrase.

4. The DNA of Claim 3 wherein said first DNA sequence includes DNA encoding an AttP site.

5. The DNA of Claim 4 wherein said first DNA sequence encodes integration into BCG.

6. The DNA of Claim 5 wherein the DNA is a plasmid including an origin of replication for E. coli.

7. The DNA of Claim 1 wherein the first DNA sequence is selected from the group consisting of Figure 4 and analogues and derivatives thereof which encode for integration into mycobacteria.

8. Mycobacteria transformed with the DNA of Claim 1.

9. BCG having foreign DNA integrated into the chromosome.

10. A vaccine comprising: the transformed mycobacteria of Claim 8; and an acceptable pharamceutical carrier.

11. A process for providing a protein, comprising:
expressing the protein in the mycobacteria of Claim 8.

AAAACACCCTCTGACCAGCCGAGCGGGCGACGGGAATCGAACCCGCGTAGCTAGTTTGGAAGATGGGTGTCTGCCGACCACA
AAAACACCCTCTGACCAGCCGAGCGGGCGACGGGAATCGAACCCGCGTAGCTAGTTTGGAAGACTAGGGCTCTACCATTGAGC
CGCACGTGGCGGTCCCTACCGAGCGGGCGACGGGAATCGAACCCGCGTAGCTAGTTTGGAAGACTAGGGCTCTACCATTGAGC
aIIP
aIIL
aIIB

Figure 1

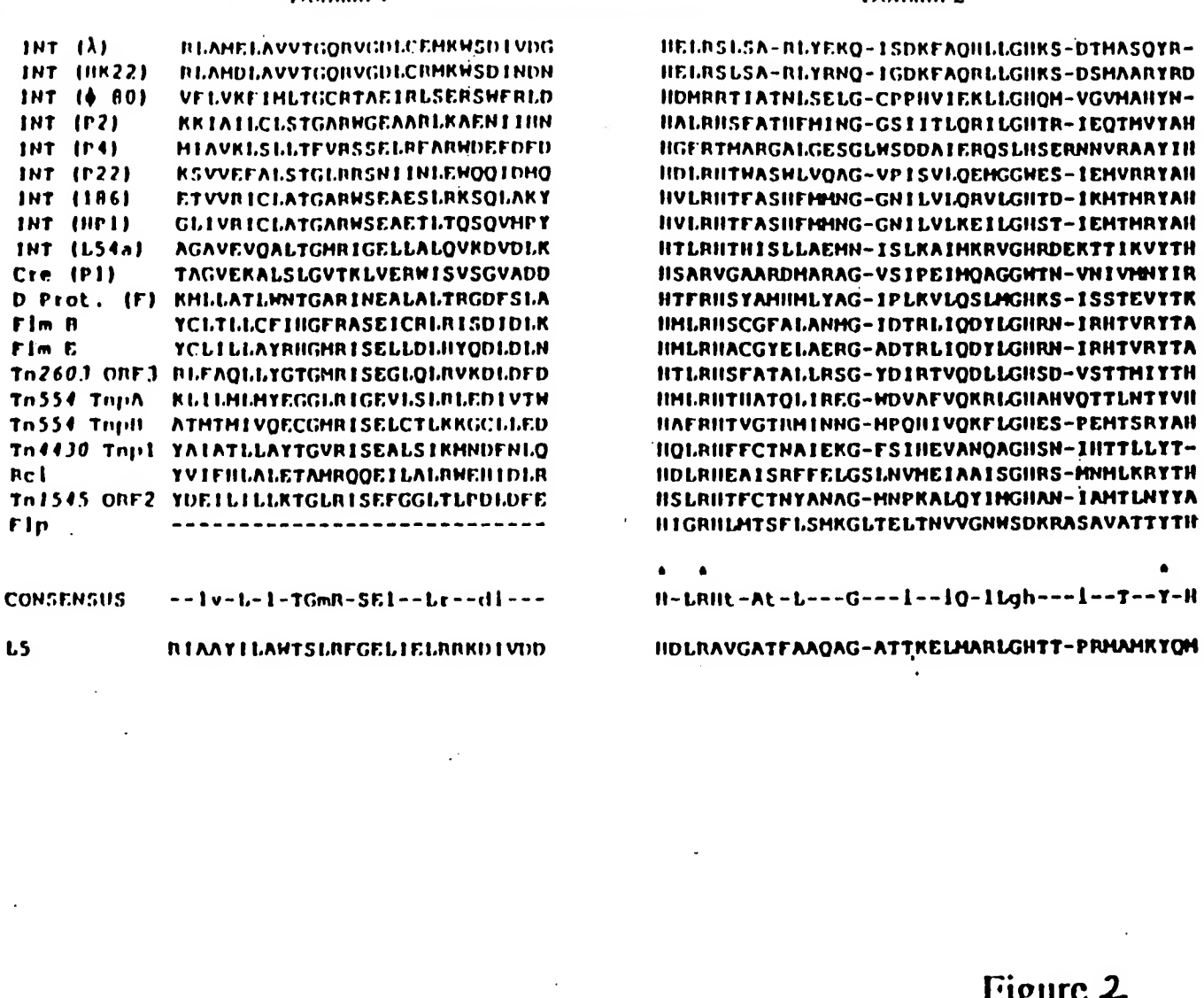

Domain 1
Domain 2
INT (λ) RLAMELAVVTGQRVGDLCEMKWSDIVDG HELRSLSA-RLYERQ-ISDKFAQHLLGHKS-DTHASQYR-
INT (HK22) RLAMDLAVVTGQRVGDLCRMKWSDINDN HELRSLSA-RLYRNQ-IGDKFAQRLLGHKS-DSHAARYRD
INT (φ 80) VFLVKFIMLTGCRTAEIRLSERSWFRLD HDMRRTIATNLSELG-CPPHVIEKLLGHQM-VGVMAHYN-
INT (P2) KKIAIICLSTGARWGEAARLKAENIIHN HALRHSFATHFMING-GSIITLQRILGHTR-IEQTMVYAH
INT (P4) MIAVKLSLLTFVRSSELRFARWDEFDFD HGFRTMARGALGESGLWSDDAIERQSLHSERNNVRAAYIH
INT (P22) KSVVEFALSTGLRRSNIINLEWQQIDMQ HDLRHTWASHLVQAG-VPISVLQEMGGWES-IEMVRRYAH
INT (186) ETVVRICLATGARWSEAESLRKSQLAKY HVLRHTFASHFMMNG-GNILVLQRVLGHTD-IKMTMRYAH
INT (HP1) GLIVRICLATGARWSEAETLTQSQVHPY HVLRHTFASHFMMNG-GNILVLKEILGHST-IEMTMRYAH
INT (L54a) AGAVEVQALTGMRIGELLALQVKDVDLK HTLRHTHISLLAEMN-ISLKAIMKRVGHRDEKTTIKVYTH
Cre (P1) TAGVEKALSLGVTKLVERWISVSGVADD HSARVGAARDMARAG-VSIPEIMQAGGWTN-VNIVMNYIR
D Prot. (F) KMLLATLWNTGARINEALALTRGDFSLA HTFRHSYAMHMLYAG-IPLKVLQSLMGHKS-ISSTEVYTK
Flm A YCLTLLCFIHGFRASEICRLRISDIDLK HMLRHSCGFALANMG-IDTRLIQDYLGHRN-IRHTVRYTA
Flm B YCLTLLLAYRHGMRISELLDLHYQDLDLN HMLRHACGYELAERG-ADTRLIQDYLGHRN-IRHTVRYTA
Tn*2601* ORF3 RLFAQLLYGTGMRISEGLQLRVKDLDFD HTLRHSFATALLRSG-YDIRTVQDLLGHSD-VSTTMIYTH
Tn*554* TnpA KLLLMLMYEGGLRIGEVLSLRLEDIVTW HMLRHTHATQLIREG-WDVAFVQKRLGHAHVQTTLNTYVH
Tn*554* TnpH ATMTMIVQECGMRISELCTLKKGCLLED HAFRHTVGTHMINNG-HPQHIVQKFLGHES-PEMTSRYAH
Tn*4430* TnpI YAIATLLAYTGVRISEALSIKMNDFNLQ HQLRHFFCTNAIEKG-FSIHEVANQAGHSN-IHTTLLYT-
RcI YVIFHLALETAMRQQEILALRWEHIDLR HDLRHEAISRFFELGSLNVMEIAAISGHRS-MNMLKRYTH
Tn*1545* ORF2 YDEILILLRTGLRISEFGGLTLPDLDFE HSLRHTFCTNYANAG-MNPKALQYIMGHAN-IAMTLNYYA
Flp ---------- HIGRHLMTSFLSMKGLTELTNVVGNWSDKRASAVATTYTH
CONSENSUS --lv-L-l-TGmR-SEl--Lr--dl--- H-LRHt-At-L---G---l--lQ-lLgh---l--T--Y-H
L5 RIAAYILAWTSLRFGELIELRRKDIVDD HDLRAVGATFAAQAG-ATTKELMARLGHTT-PRMAMKYQH

Figure 2

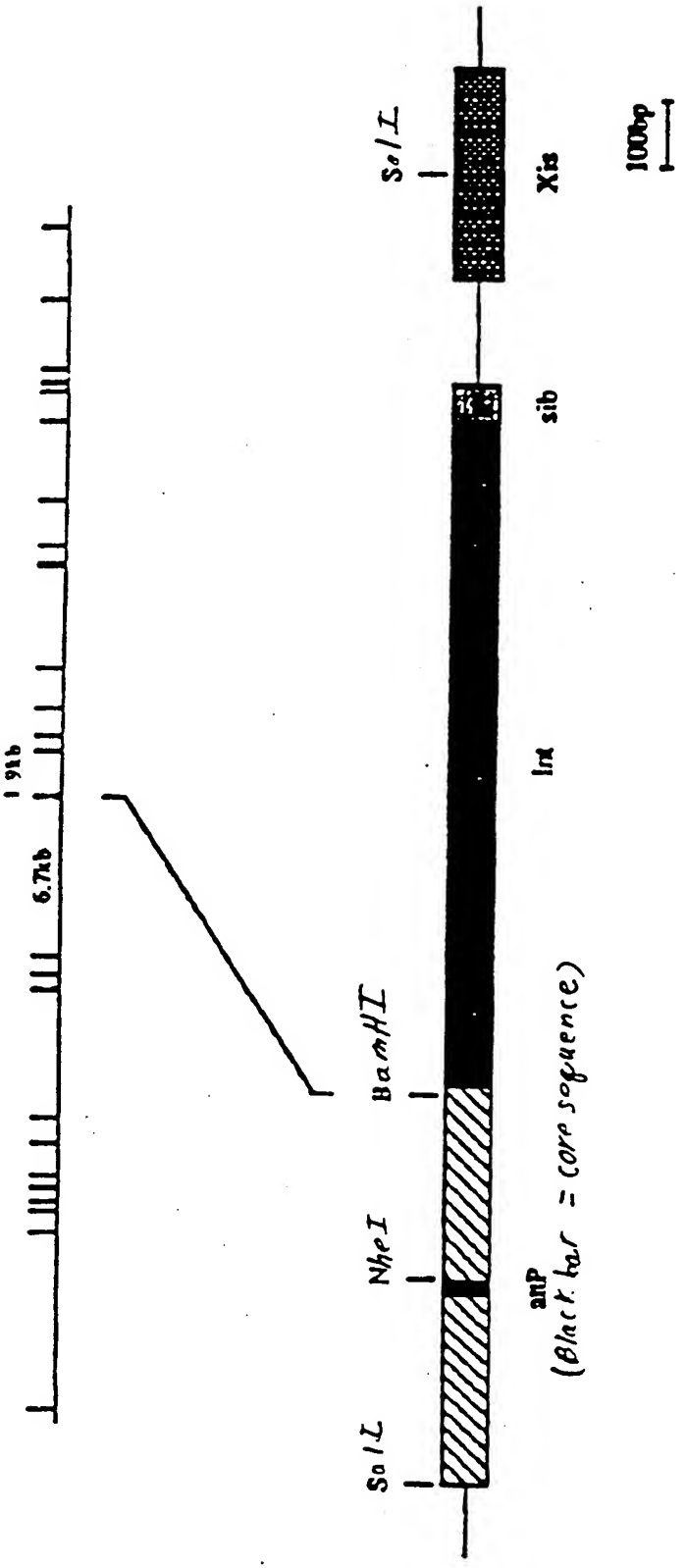

L5 BamHI sites
5kb
19kb
6.7kb
SalI
NheI
BamHI
SalI
Xis
sib
int
100bp
amP
(black bar = core sequence)

Figure 3

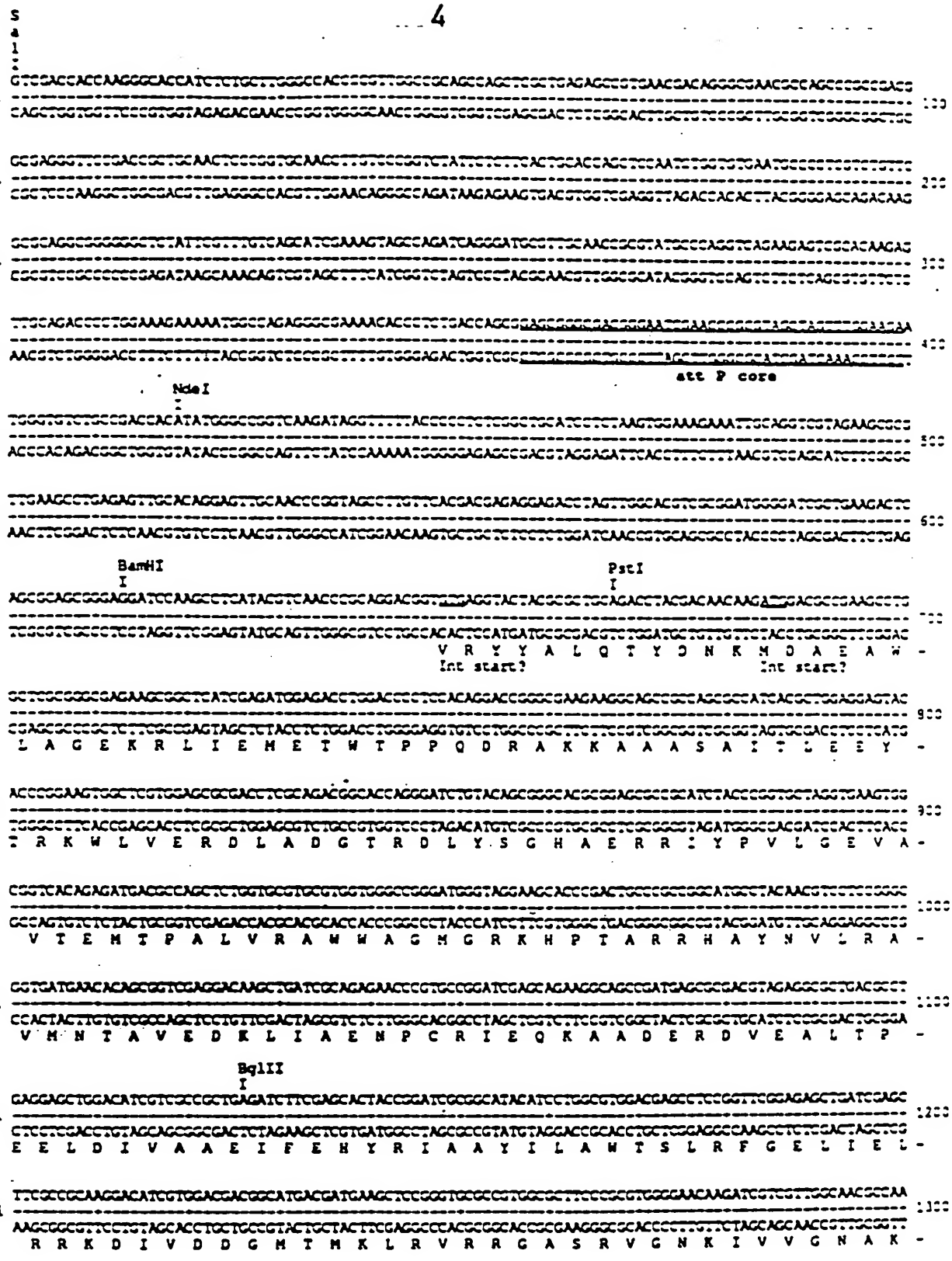
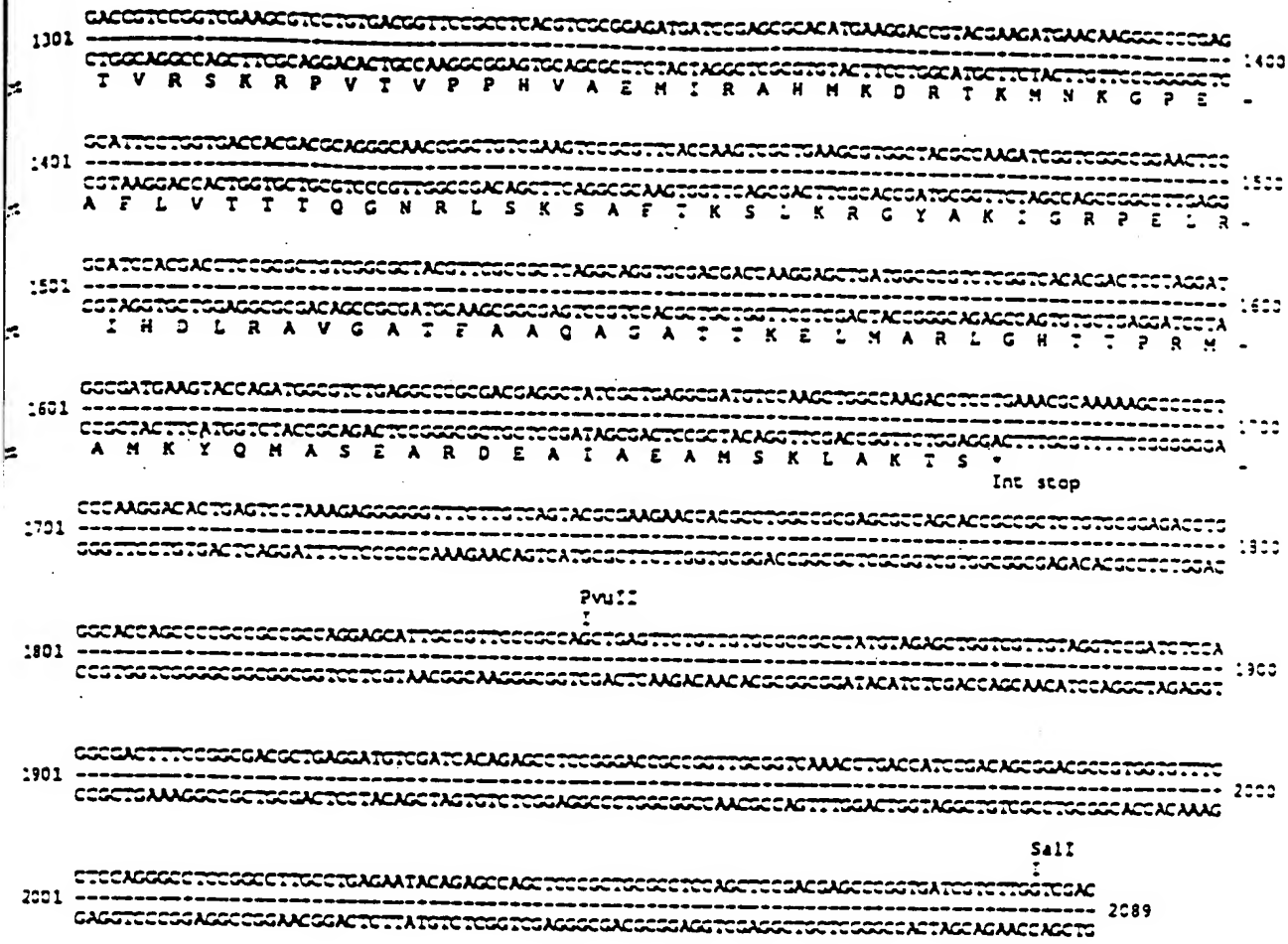


Figure 4


Int stop
PvuII
SalI

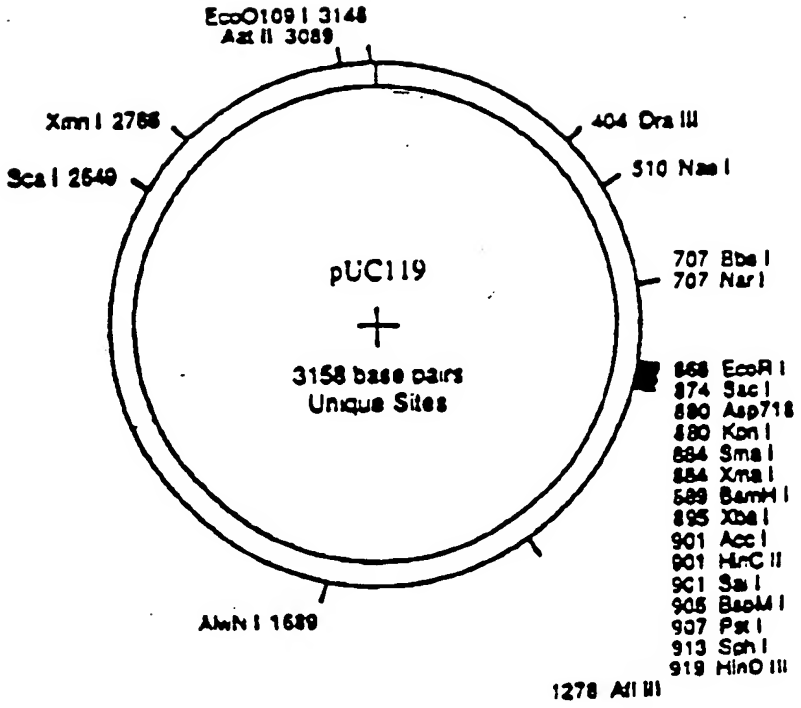
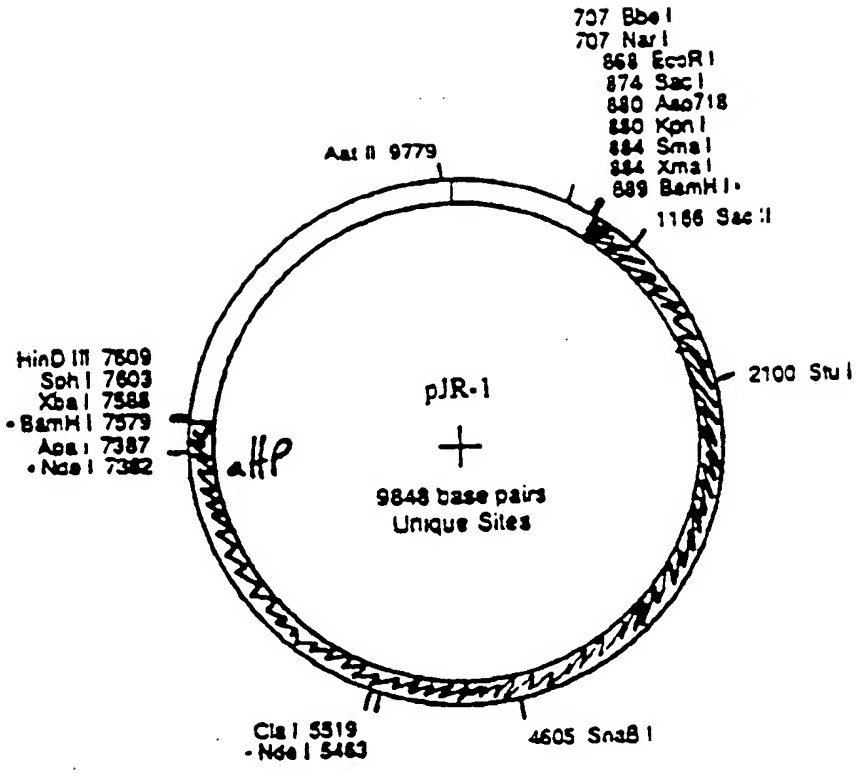
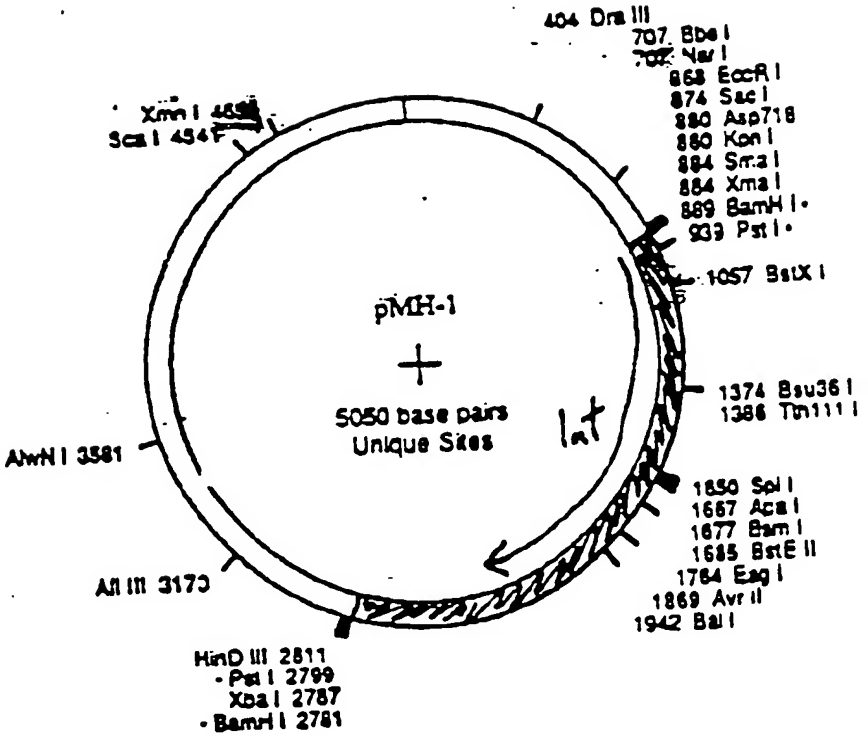

EcoO109 I 3148
Aat II 3089
Xmn I 2786
Sca I 2649
404 Dra III
510 Nae I
707 Bbe I
707 Nar I
pUC119
3158 base pairs
Unique Sites
868 EcoR I
874 Sac I
880 Asp718
880 Kpn I
884 Sma I
884 Xma I
889 BamH I
895 Xba I
901 Acc I
901 HinC II
901 Sal I
905 BspM I
907 Pst I
913 Sph I
919 HinD III
1278 Afl III
AlwN I 1689

Figure 5



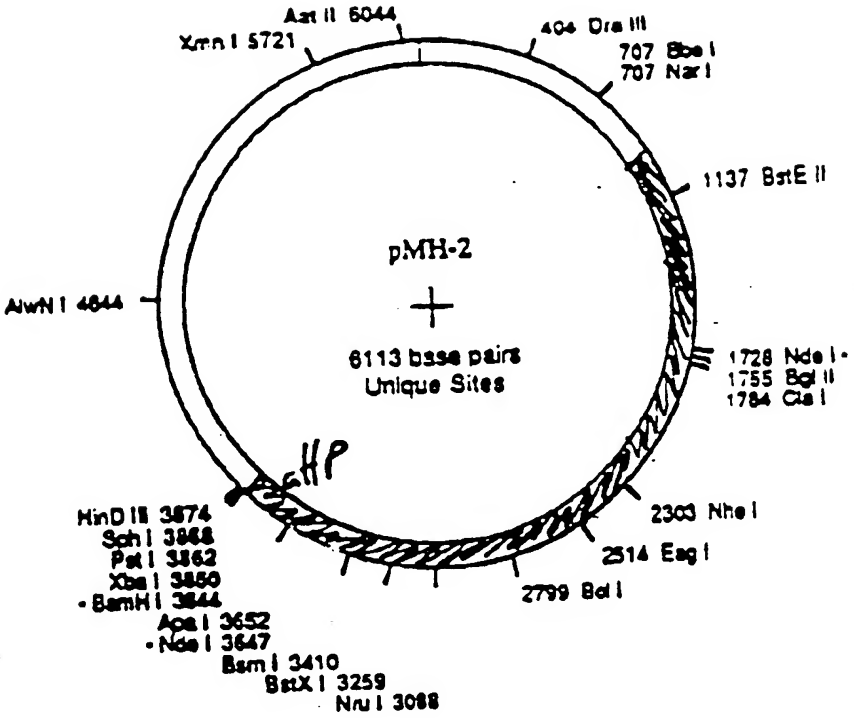
Shaded portion = LS DNA

Figure 6



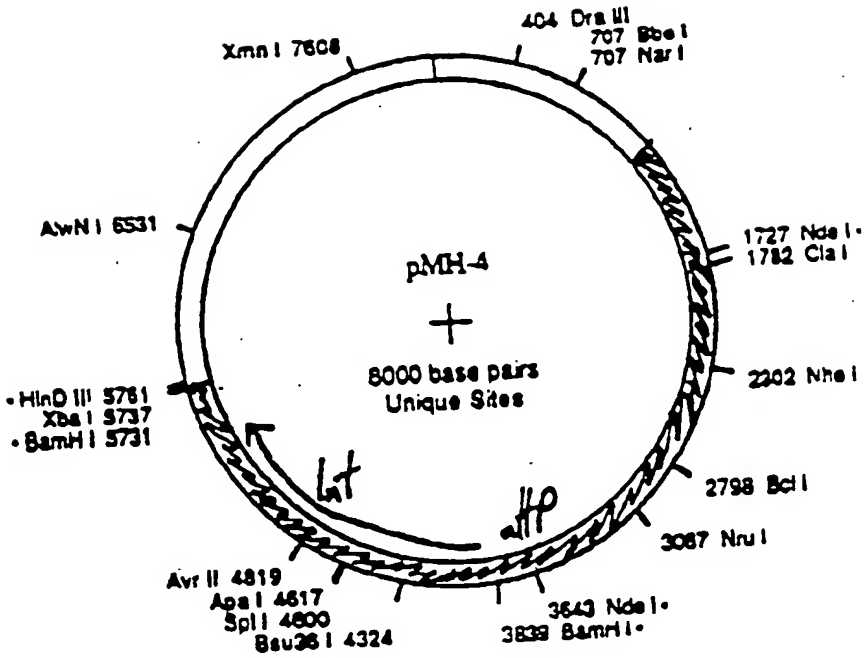
Shaded portion = LS⁻ DNA

Figure 7



Shaded portion = LS DNA

Figure 8



Shaded portion = L5 DNA

Figure 9

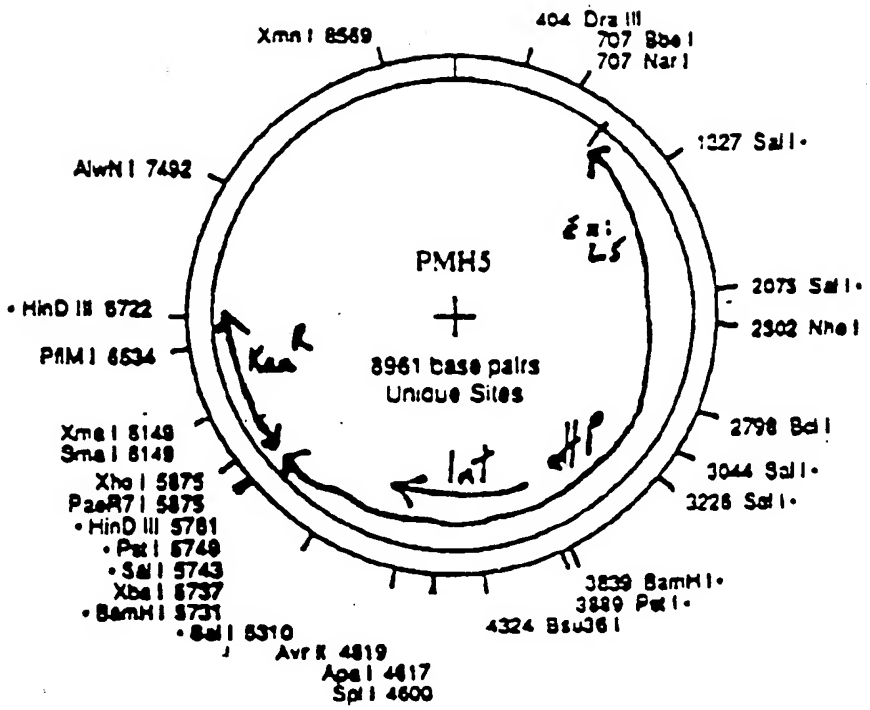
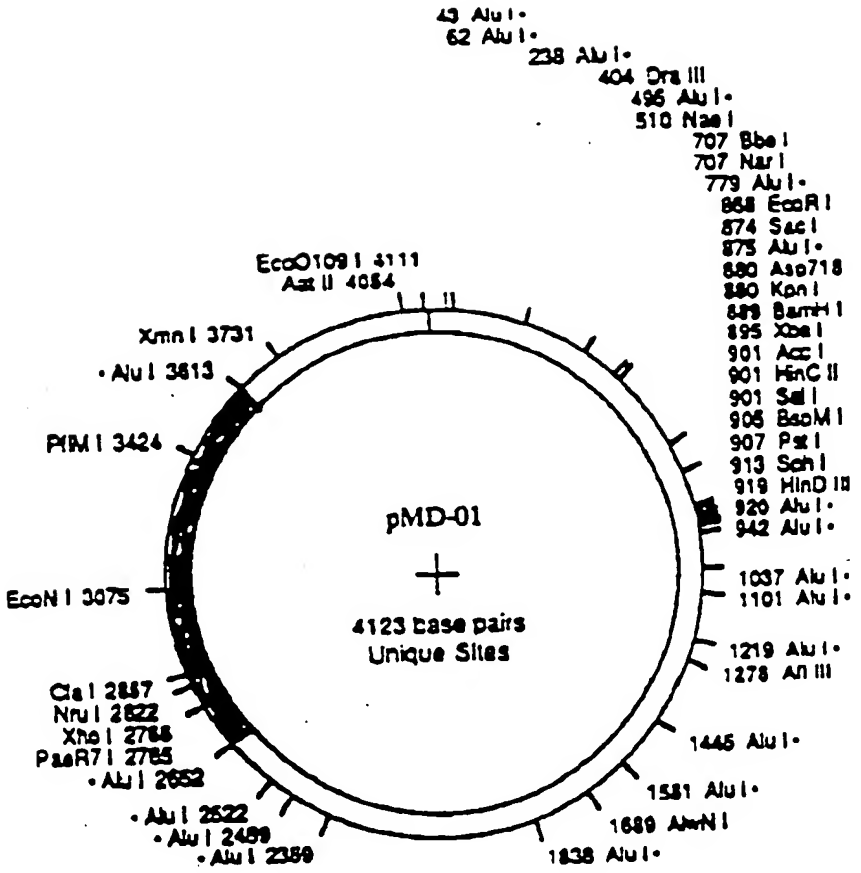

PMH5
8961 base pairs
Unique Sites
Kan R
lacI
404 Dra III
707 Bbe I
707 Nar I
1227 Sal I
2073 Sal I
2302 Nhe I
2798 Bcl I
3044 Sal I
3226 Sal I
3839 BamH I
3889 Pst I
4324 Bsu36 I
Avr II 4819
Apa I 4617
Spl I 4600
Sal I 5310
BamH I 5731
Xba I 5737
Sal I 5743
Pst I 5749
HinD III 5781
PaeR7 I 5875
Xho I 5875
Sma I 6149
Xma I 6149
PflM I 6534
HinD III 6722
AlwN I 7492
Xmn I 8569

Figure 10



Shaded portion = Kan^R cassette

Figure 11

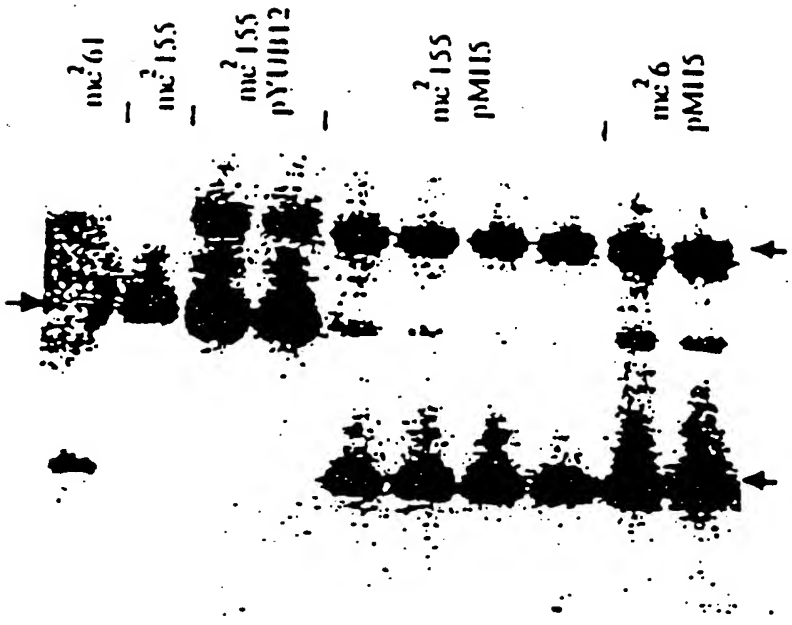
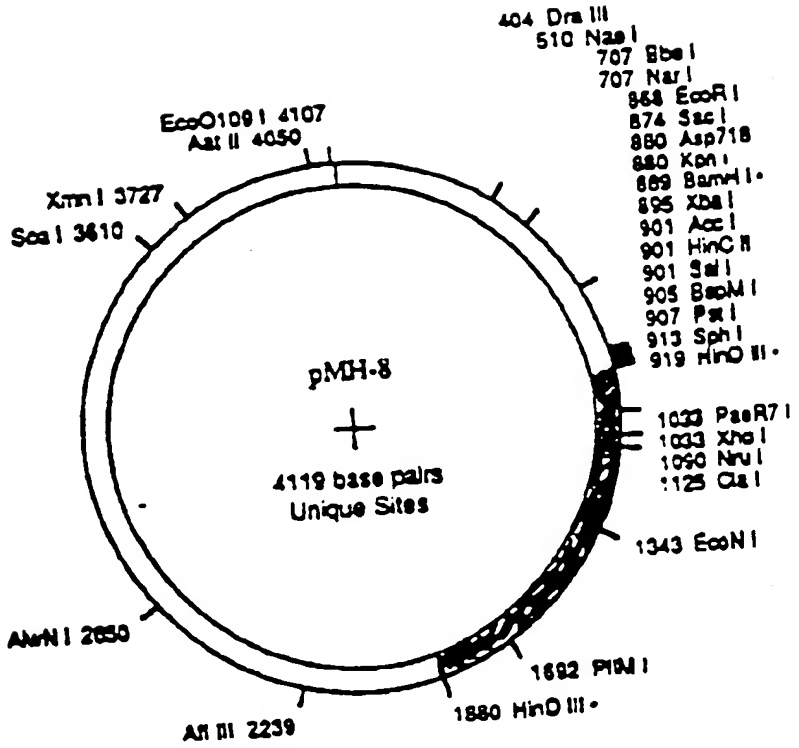


FIGURE 12



Shaded portion = Kan^R cassette

Figure 13

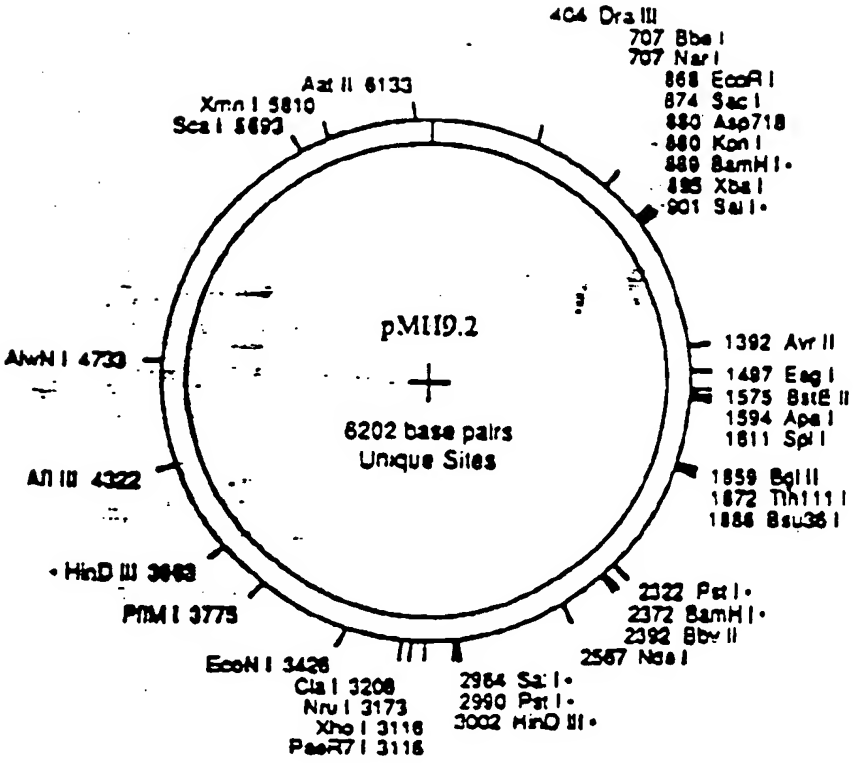

404 Dra III
707 Bbe I
707 Nar I
868 EcoR I
874 Sac I
880 Asp718
880 Kon I
889 BamH I
895 Xba I
901 Sal I
1392 Avr II
1497 Eag I
1575 BstE II
1594 Apa I
1611 Spl I
1859 Bgl II
1872 Tth111 I
1886 Bsu36 I
2322 Pst I
2372 BamH I
2392 Bbv II
2567 Nde I
2984 Sal I
2990 Pst I
3002 HinD III
3116 Xho I
3116 PaeR7 I
3126 Cla I
3173 Nru I
3426 EcoN I
3775 PflM I
4322 Afl III
4733 AlwN I
5693 Sca I
5810 Xmn I
6133 Asl II
pMII9.2
6202 base pairs
Unique Sites

Figure 14

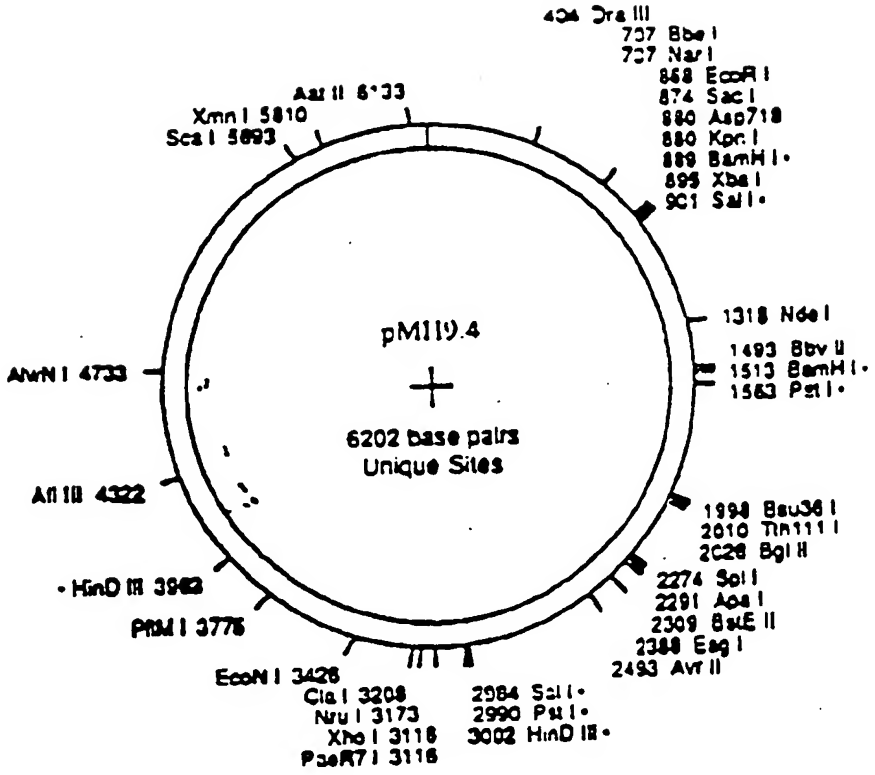

pM119.4
6202 base pairs
Unique Sites
737 Bbe I
737 Nar I
868 EcoR I
874 Sac I
880 Asp718
880 Kpn I
889 BamH I
895 Xba I
901 Sal I
1318 Nde I
1493 Bbv II
1513 BamH I
1563 Pst I
1998 Bsu36 I
2010 Tth111 I
2026 Bgl II
2274 Sal I
2291 Aca I
2309 BstE II
2388 Esg I
2493 Avr II
2984 Sal I
2990 Pst I
3002 HinD III
3116 Xho I
3116 PaeR7 I
3173 Nru I
3208 Cla I
3426 EcoN I
3776 PflM I
3962 HinD III
4322 Afl III
4733 AlwN I
5693 Sca I
5810 Xmn I
6133 Asc II

Figure 15

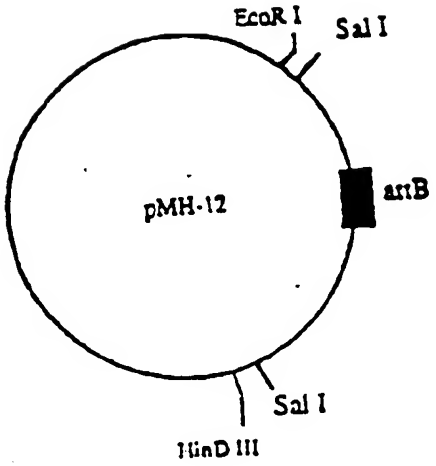


Figure 16

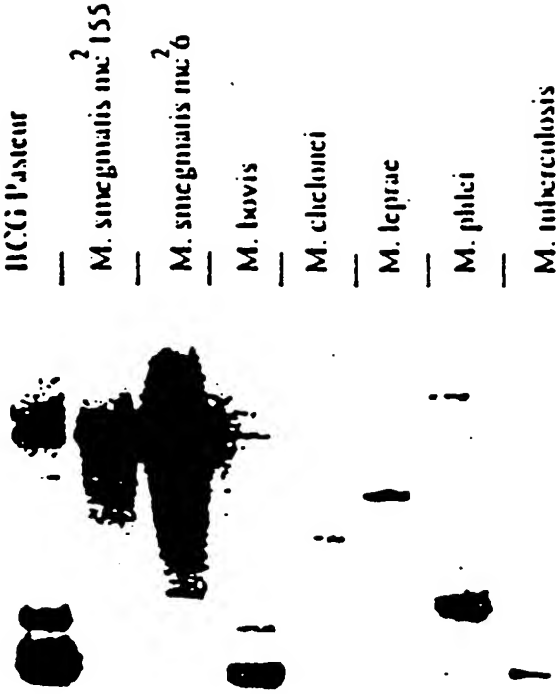

BCG Pasteur
M. smegmatis mc² 155
M. smegmatis mc² 6
M. bovis
M. chelonei
M. leprae
M. phlei
M. tuberculosis

Figure 17

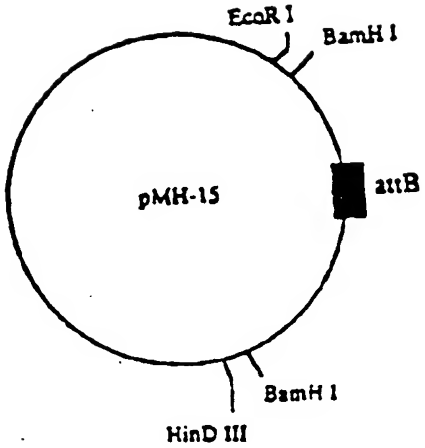


Figure 18

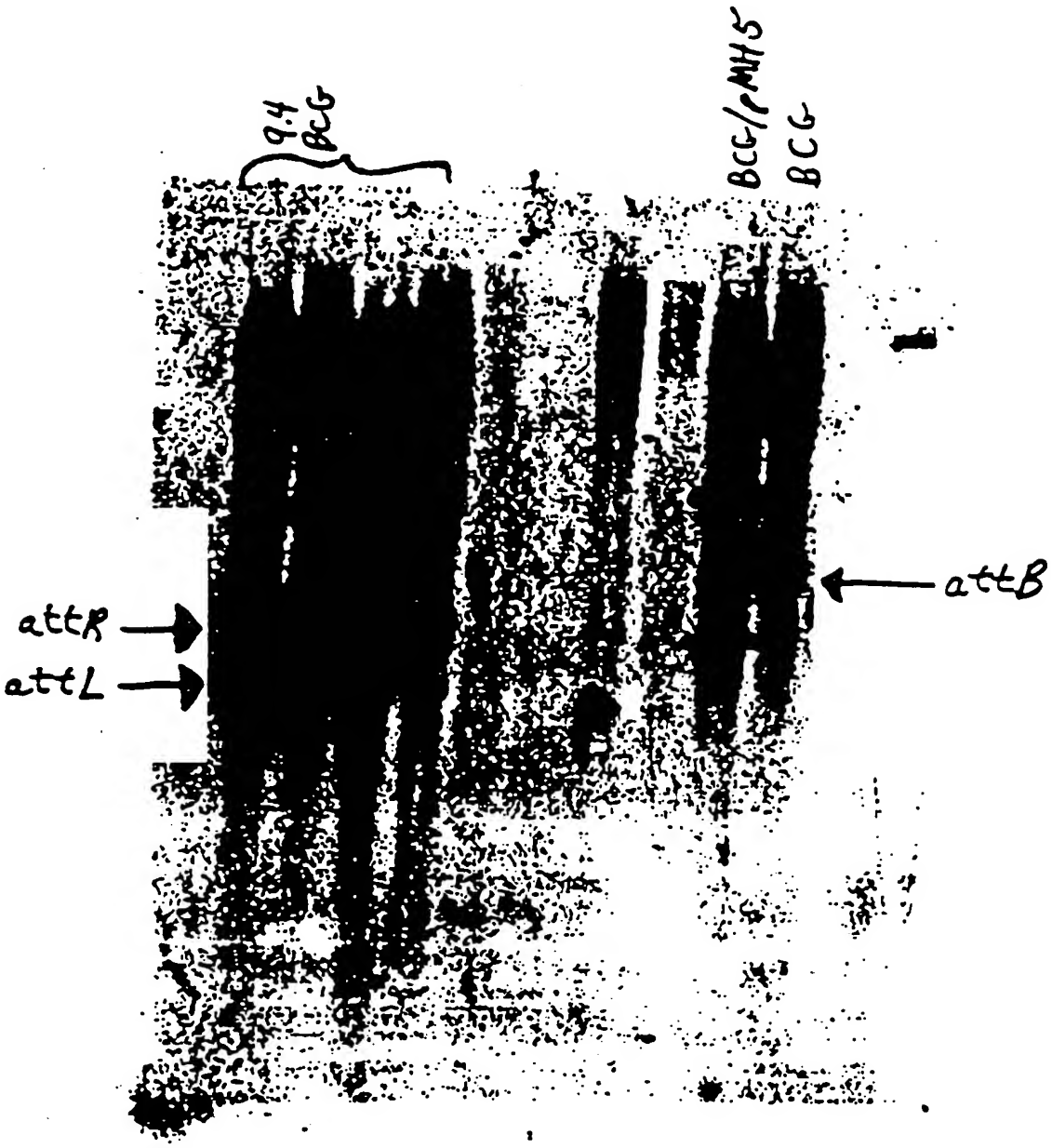


Figure 19

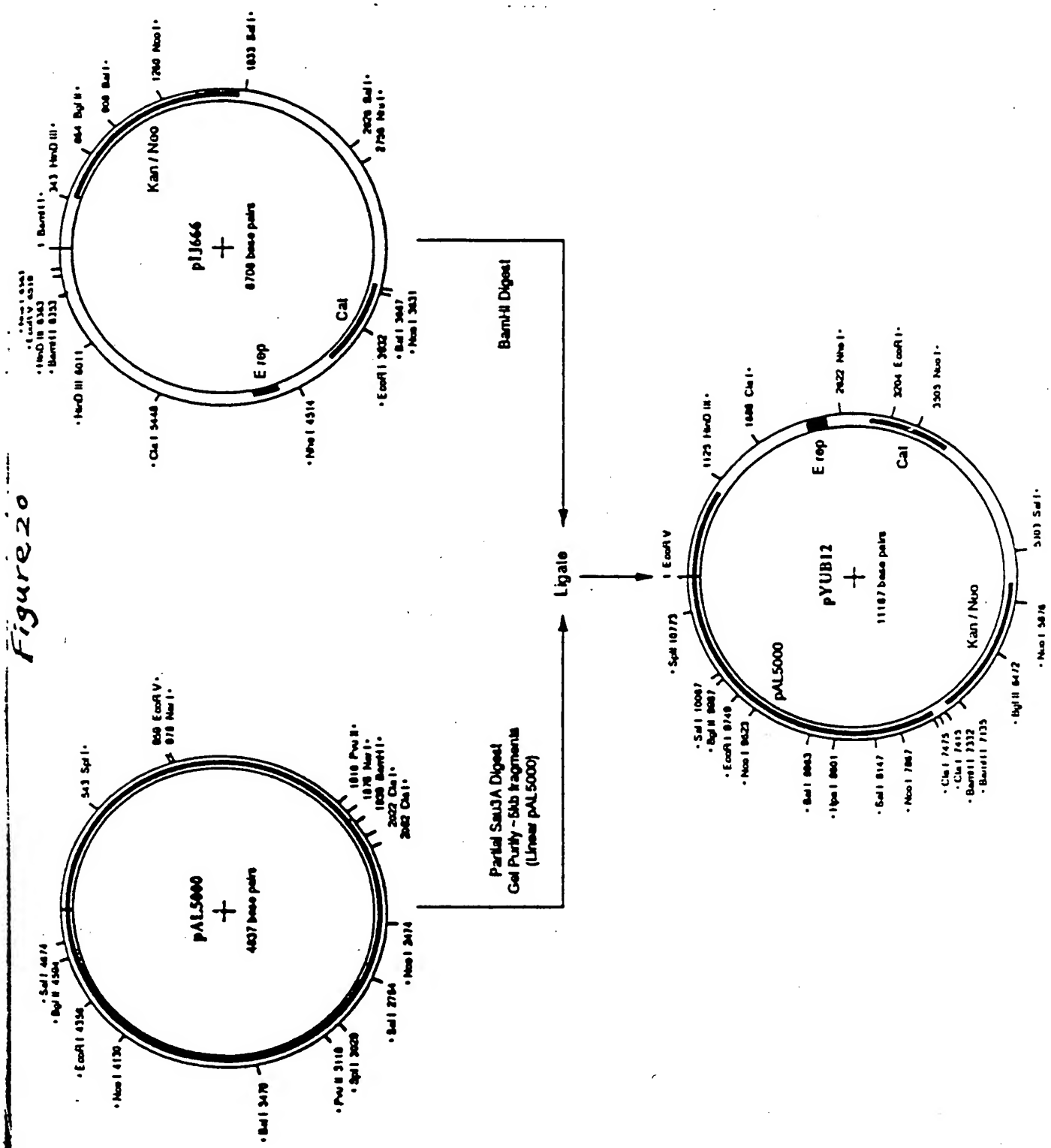

pIJ666
+
8708 base pairs
Kan / Neo
E rep
Cat
BamHI Digest
Partial Sau3A Digest
Gel Purify ~5kb fragments
(Linear pAL5000)
Ligate
pAL5000
+
4837 base pairs
pYUB12
+
11187 base pairs
pAL5000
Kan / Neo
E rep
Cat

Figure 20

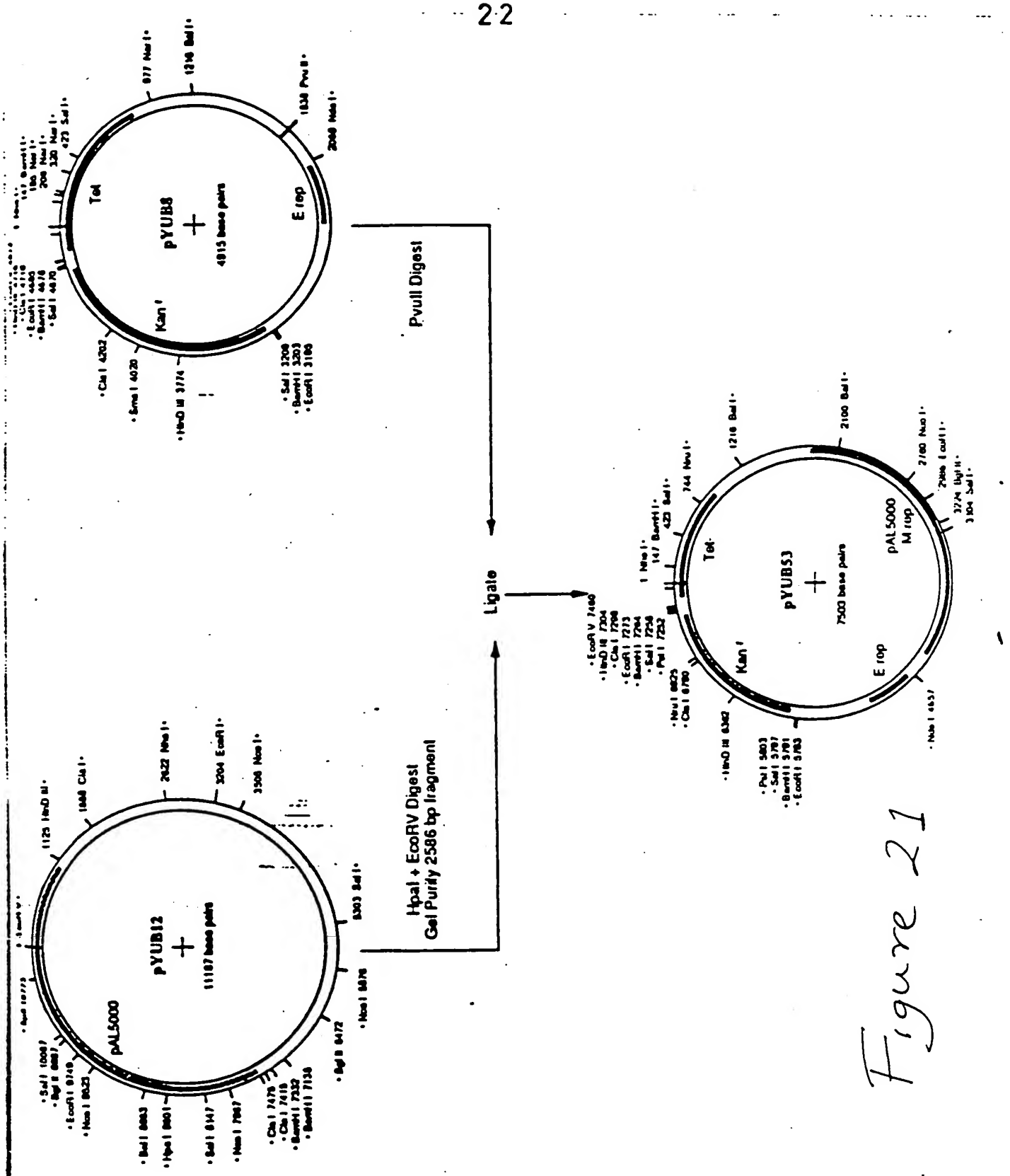

pYUB8
4815 base pairs
Tet
Kan r
E rop
PvuII Digest
pYUB12
11187 base pairs
pAL5000
HpaI + EcoRV Digest
Gel Purify 2586 bp fragment
Ligate
pYUB53
7500 base pairs
pAL5000
M rop
Tet
Kan r
E rop

Figure 21

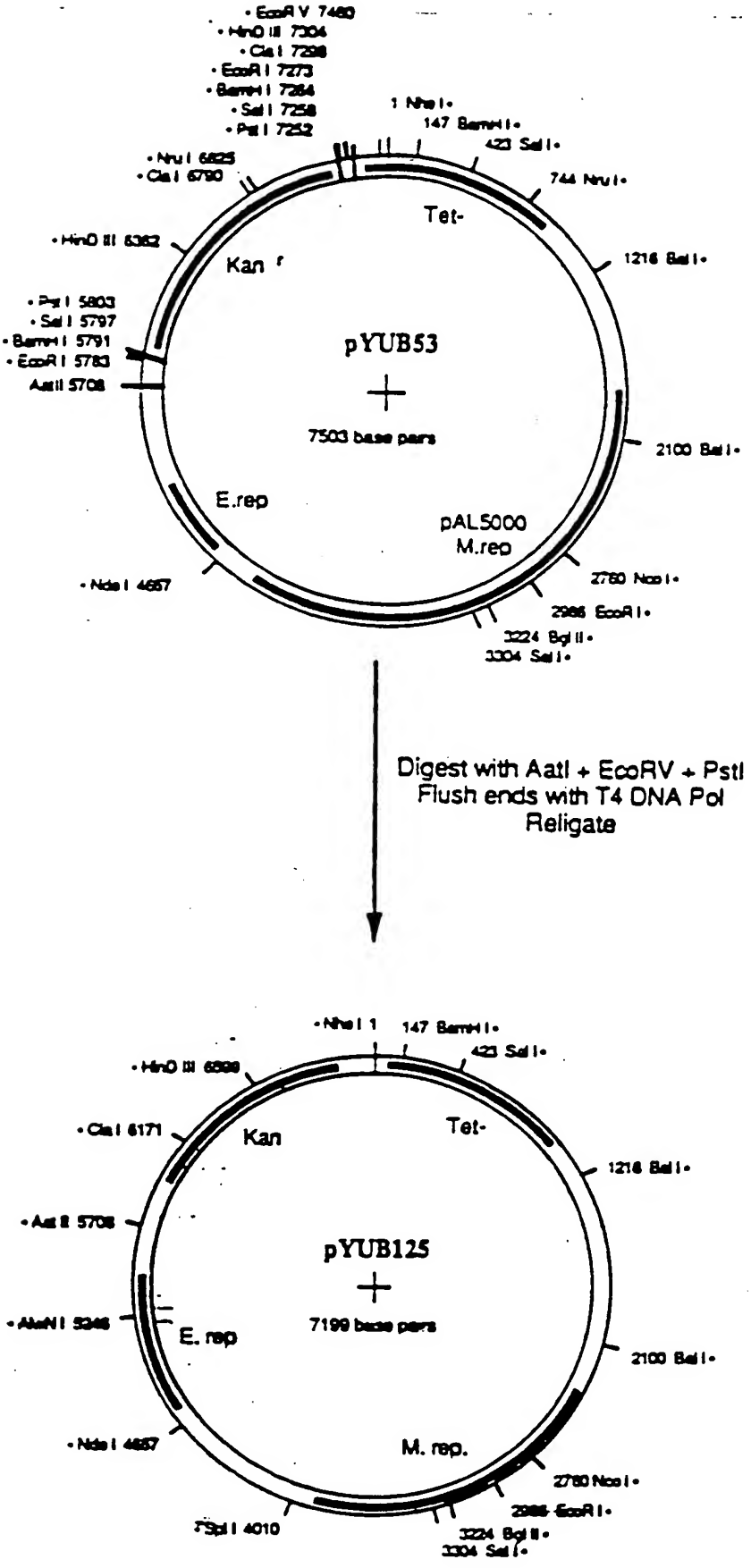

• EcoR V 7460
• Hind III 7304
• Cla I 7298
• EcoR I 7273
• BamH I 7264
• Sal I 7258
• Pst I 7252
• Nru I 6825
• Cla I 6790
• Hind III 6362
• Pst I 5803
• Sal I 5797
• BamH I 5791
• EcoR I 5783
• Aat II 5708
• Nde I 4657
1 Nhe I •
147 BamH I •
423 Sal I •
744 Nru I •
1216 Bal I •
2100 Bal I •
2760 Nco I •
2986 EcoR I •
3224 Bgl II •
3304 Sal I •
Tet-
Kan r
pYUB53
7503 base pairs
E.rep
pAL5000
M.rep
Digest with AatI + EcoRV + PstI
Flush ends with T4 DNA Pol
Religate
• Nhe I 1
147 BamH I •
423 Sal I •
• Hind III 6999
• Cla I 6171
• Aat II 5708
• Ase I 5346
• Nde I 4657
• Spl I 4010
Kan
Tet-
pYUB125
7199 base pairs
E. rep
M. rep.
1216 Bal I •
2100 Bal I •
2760 Nco I •
2986 EcoR I •
3224 Bgl II •
3304 Sal I •

Figure 22

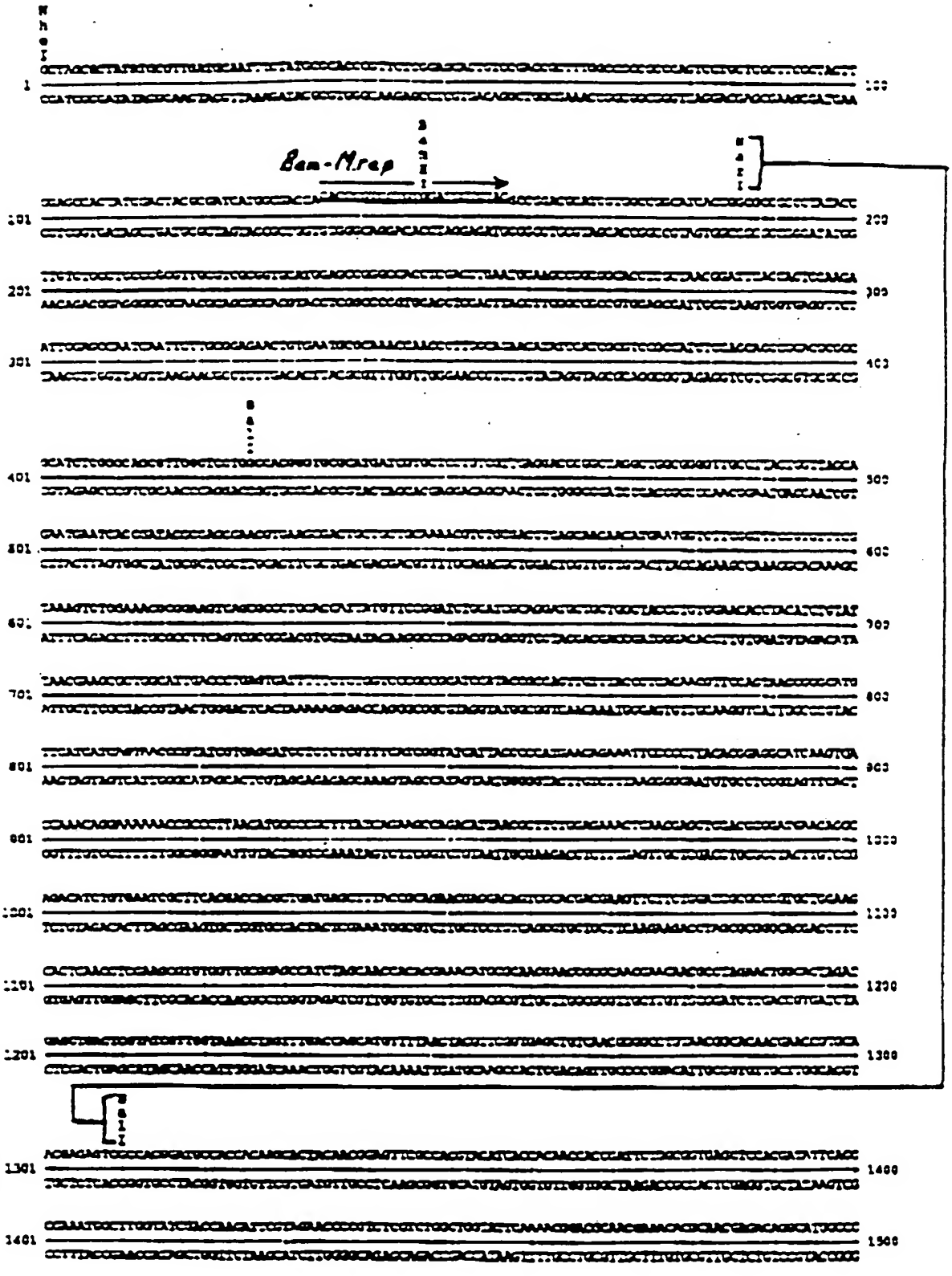
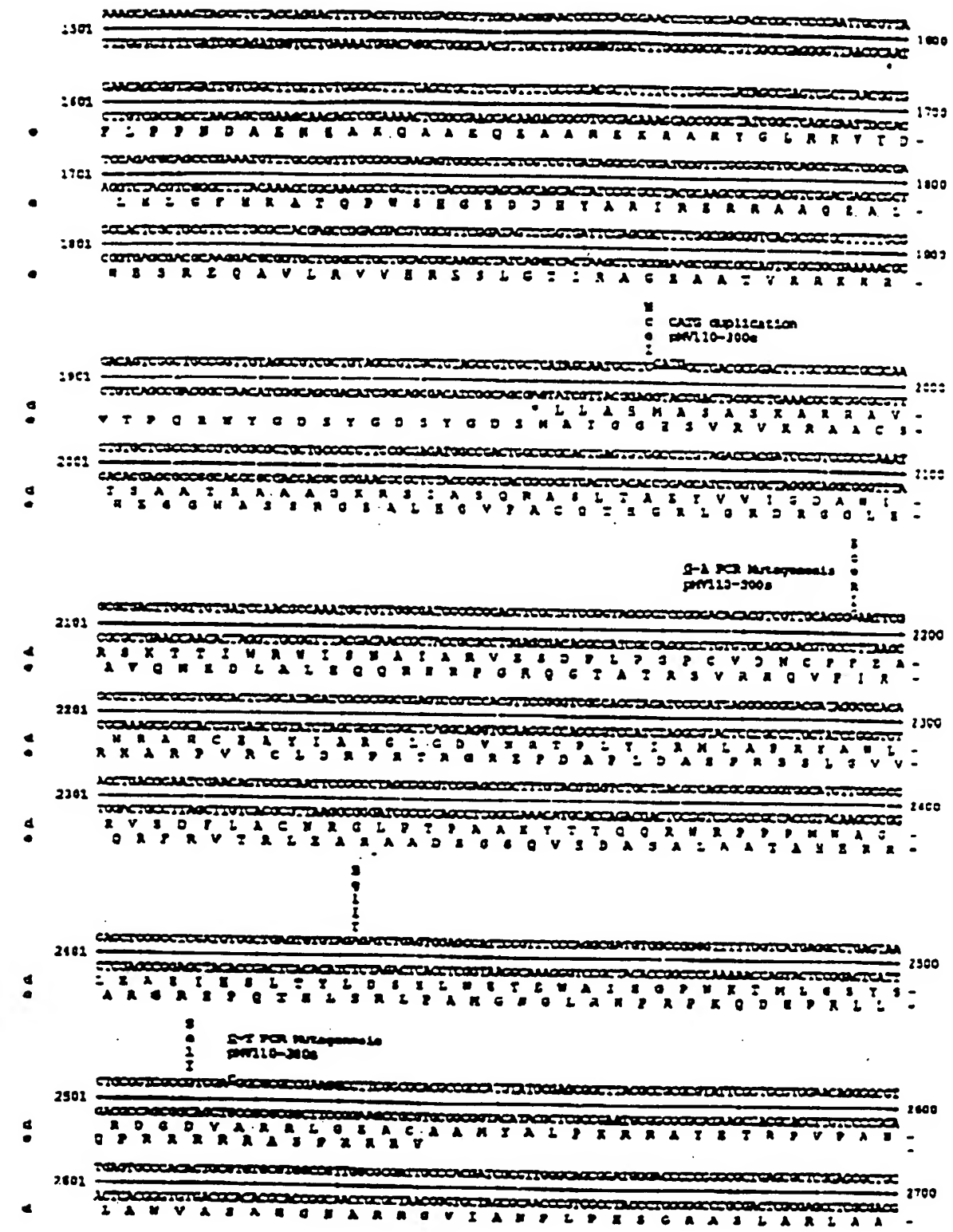
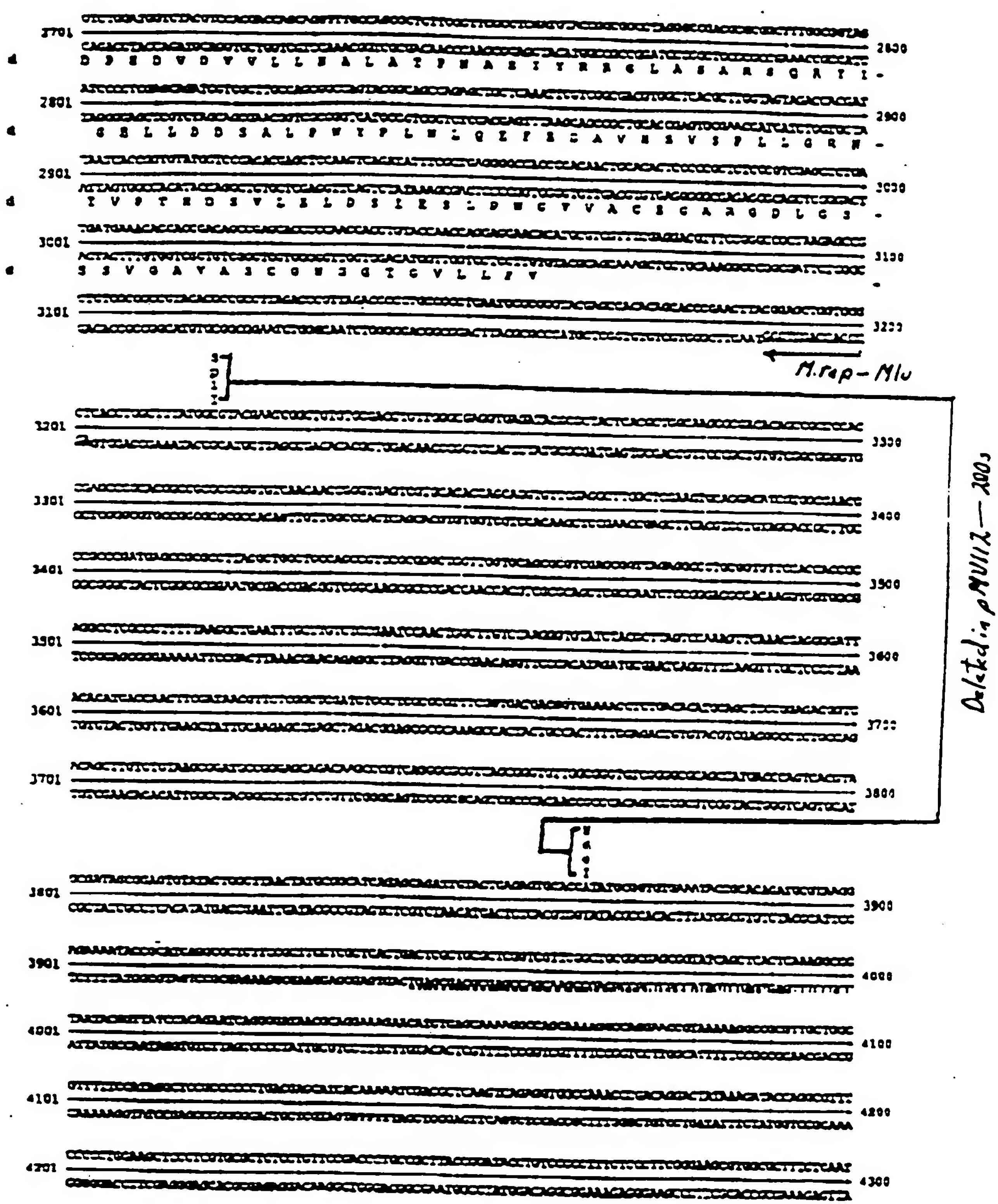
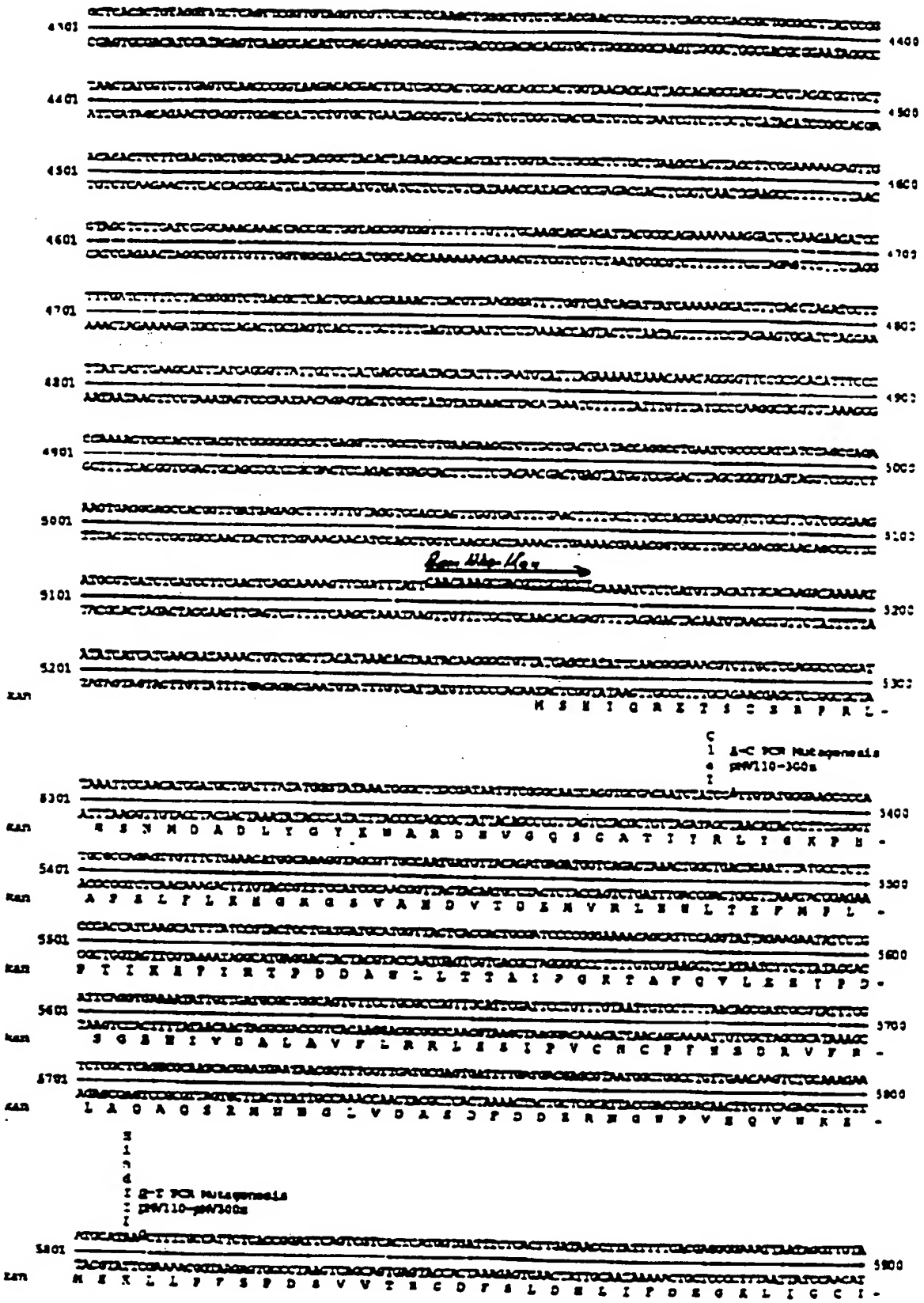
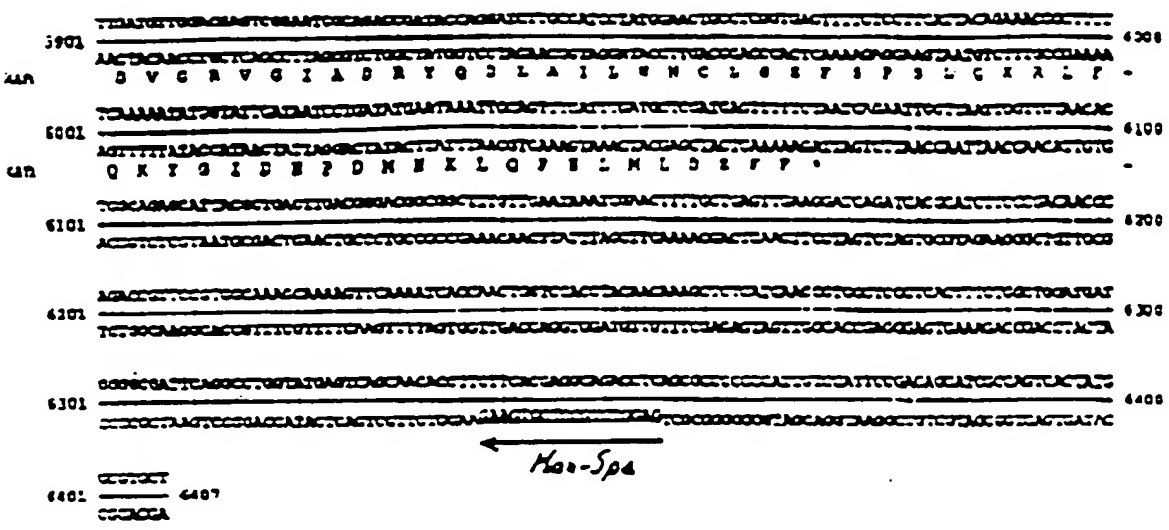


Figure 24


CAT2 duplication
pMV110-300s
Ω-1 PCR Mutagenesis
pMV110-300s
Ω-2 PCR Mutagenesis
pMV110-300s


2701
2800
2801
2900
2901
3000
3001
3100
3101
3200
M.rep-Mlu
3201
3300
3301
3400
3401
3500
3501
3600
3601
3700
3701
3800
3801
3900
3901
4000
4001
4100
4101
4200
4201
4300




Hae-Sp4

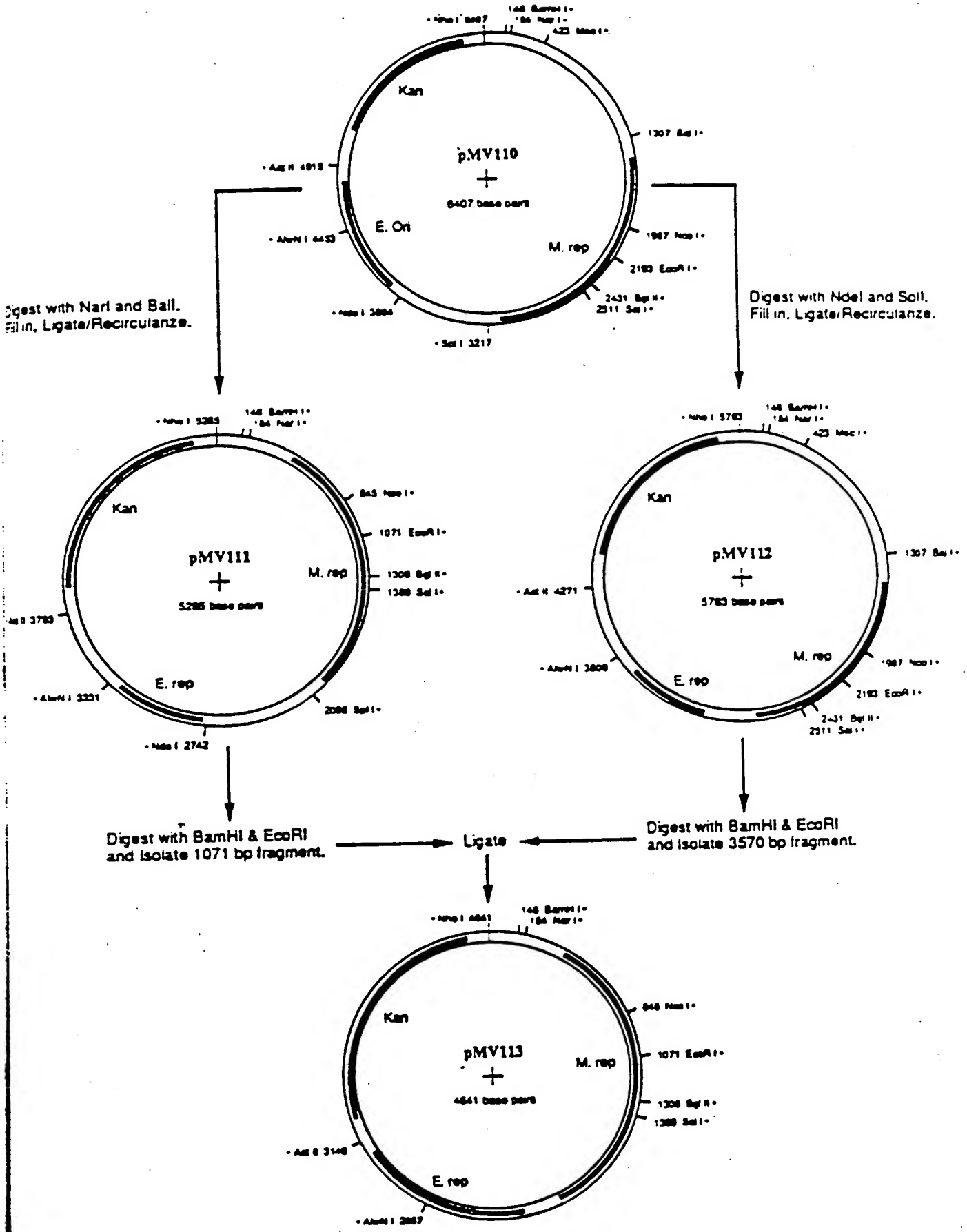

pMV110
6407 base pairs
Kan
E. Ori
M. rep
Digest with NarI and BalI.
Fill in, Ligate/Recirculanze.
Digest with NdeI and SolI.
Fill in, Ligate/Recirculanze.
pMV111
5285 base pairs
Kan
E. rep
M. rep
pMV112
5783 base pairs
Kan
E. rep
M. rep
Digest with BamHI & EcoRI
and isolate 1071 bp fragment.
Ligate
Digest with BamHI & EcoRI
and isolate 3570 bp fragment.
pMV113
4641 base pairs
Kan
E. rep
M. rep

Figure 23

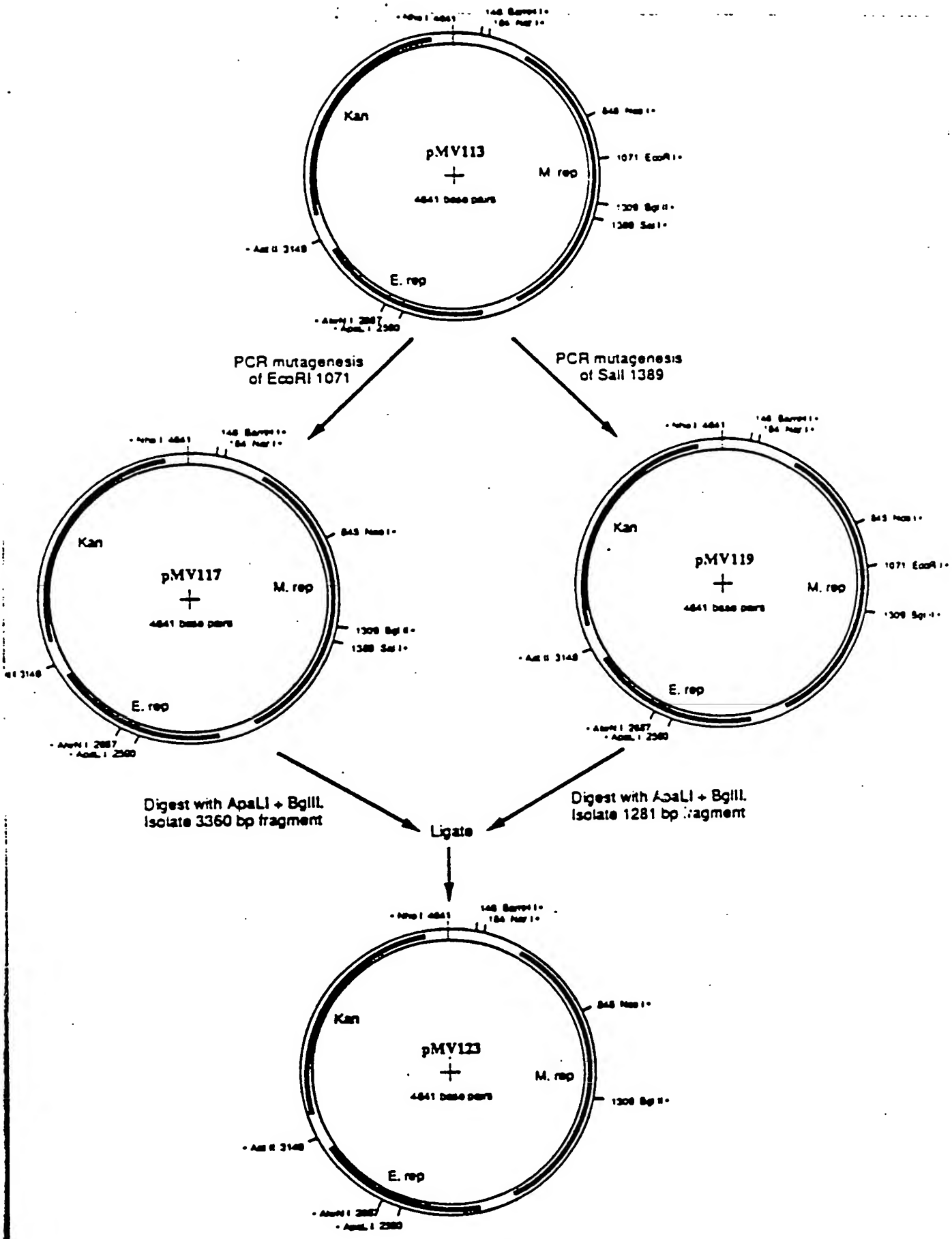

Kan
pMV113
4841 base pairs
M. rep
E. rep
PCR mutagenesis of EcoRI 1071
PCR mutagenesis of SalI 1389
Kan
pMV117
4841 base pairs
M. rep
E. rep
Kan
pMV119
4841 base pairs
M. rep
E. rep
Digest with ApaLI + BglII. Isolate 3360 bp fragment
Digest with ApaLI + BglII. Isolate 1281 bp fragment
Ligate
Kan
pMV123
4841 base pairs
M. rep
E. rep

Figure 26

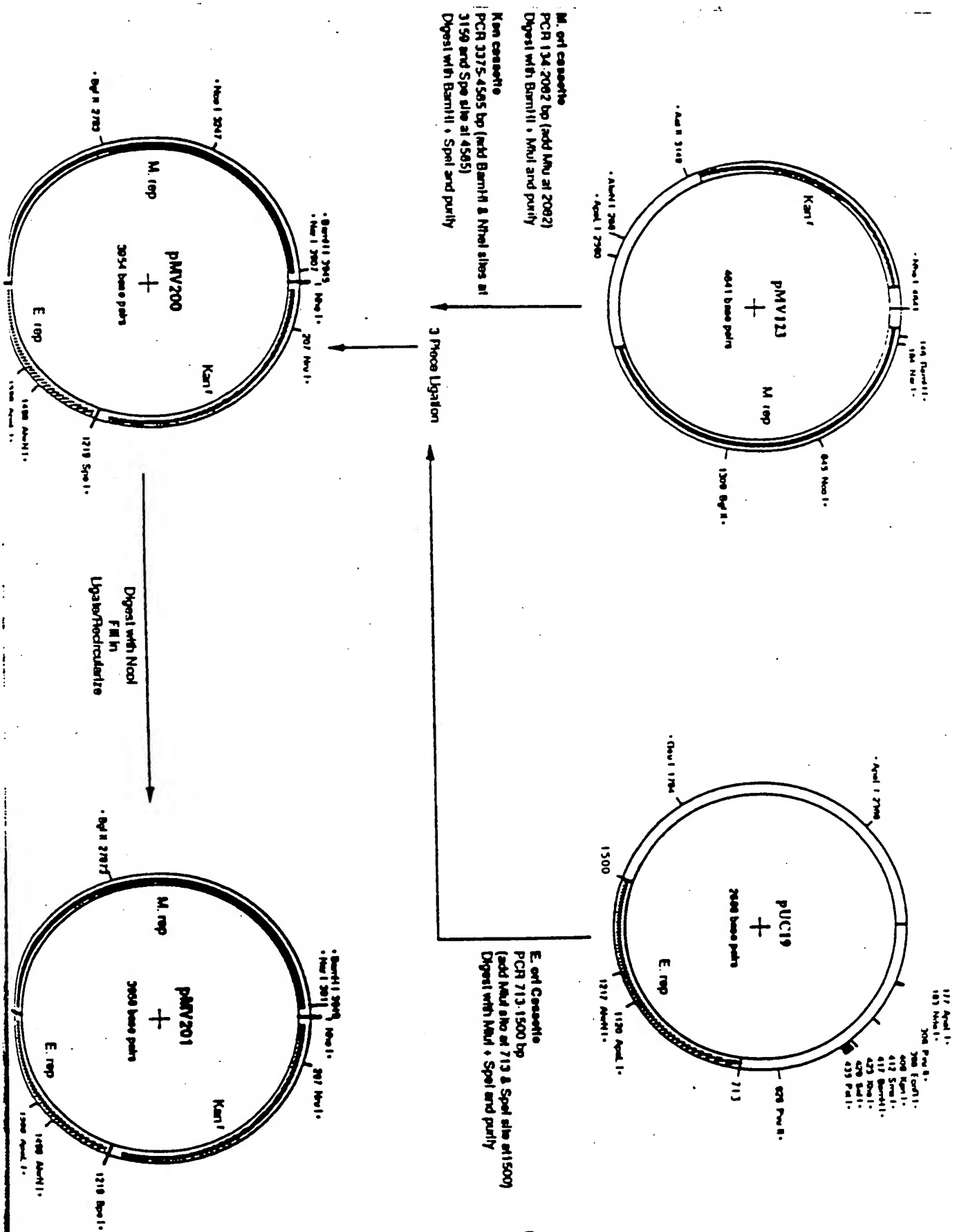

pMV123
4641 base pairs
M rep
Kan'
pMV200
3954 base pairs
M rep
E rep
Kan'
pUC19
2688 base pairs
E rep
pMV201
3969 base pairs
M rep
E rep
Kan'
M. ori cassette
PCR 134-2082 bp (add MIu at 2082)
Digest with BamHI + MIuI and purify
Kan cassette
PCR 3375-4565 bp (add BamHI & NheI sites at 3159 and Spe site at 4565)
Digest with BamHI + SpeI and purify
3 Piece Ligation
E. ori Cassette
PCR 713-1500 bp
(add MIuI site at 713 & SpeI site at1500)
Digest with MIuI + SpeI and purify
Digest with NcoI
Fill in
Ligate/Recircularize

Figure27

Synthetic Multiple Cloning Site (MCS) + Strand

GAA GGC GCG GCC GCG GTA CCA GAT CTT TAA ATC TAG ATA TCC ATG GAT
CCA GCT GCA GAA TTC GAA GCT TAT CGA TGT CGA CGT AGT TAA CTA GCG
TAC GAT CGA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG
ACT GGG CCT TTC GTT TTA TCT GTT GTT TGT CCG GCC ATC ATG GCC GCG
GTG ATC AGC TAG TAC G

Figure 28

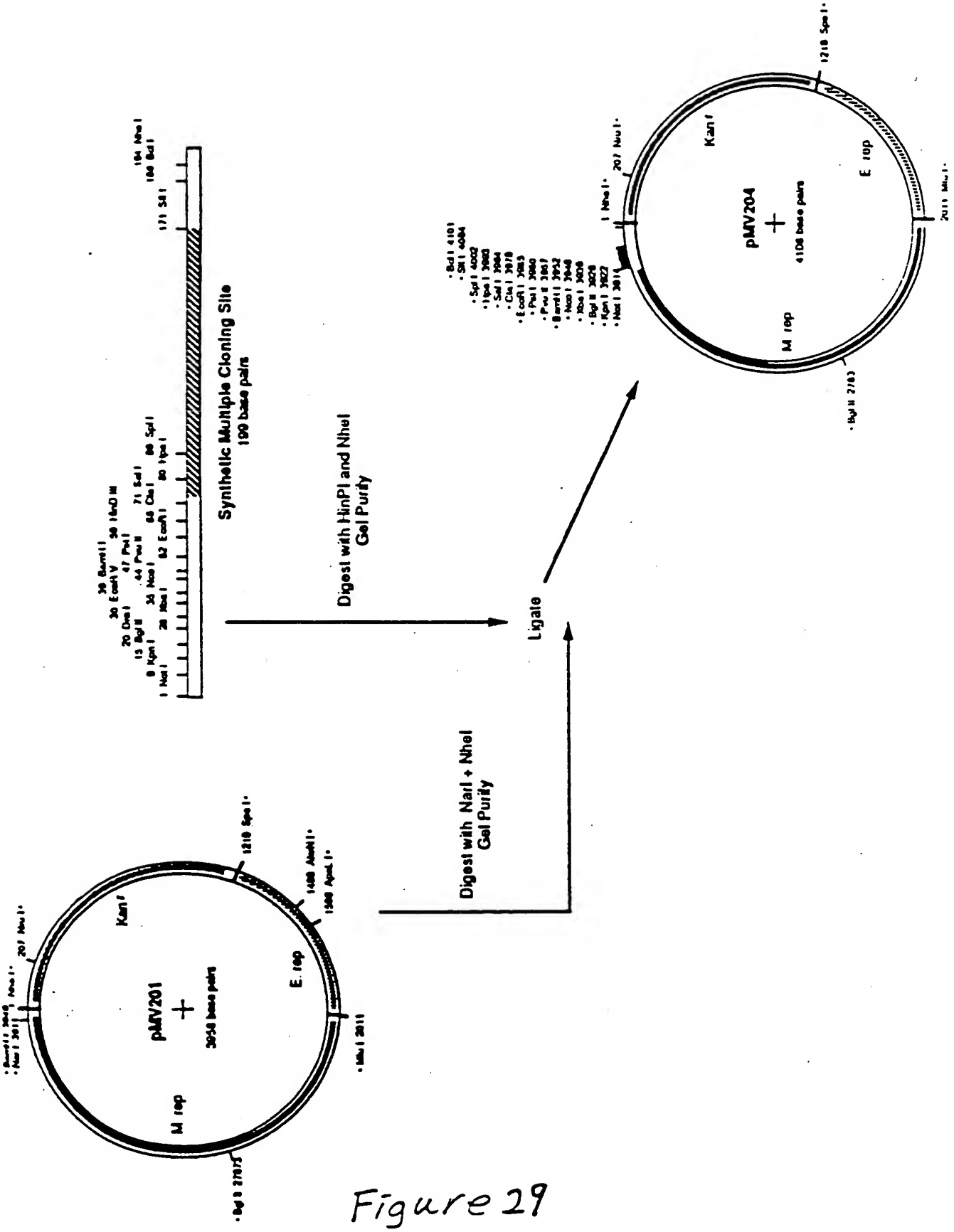

Synthetic Multiple Cloning Site
190 base pairs
Digest with HinPI and NheI
Gel Purify
Ligate
Digest with NarI + NheI
Gel Purify
pMV204
4106 base pairs
pMV201
3954 base pairs
Kan r
E. rep
M rep

Figure 29

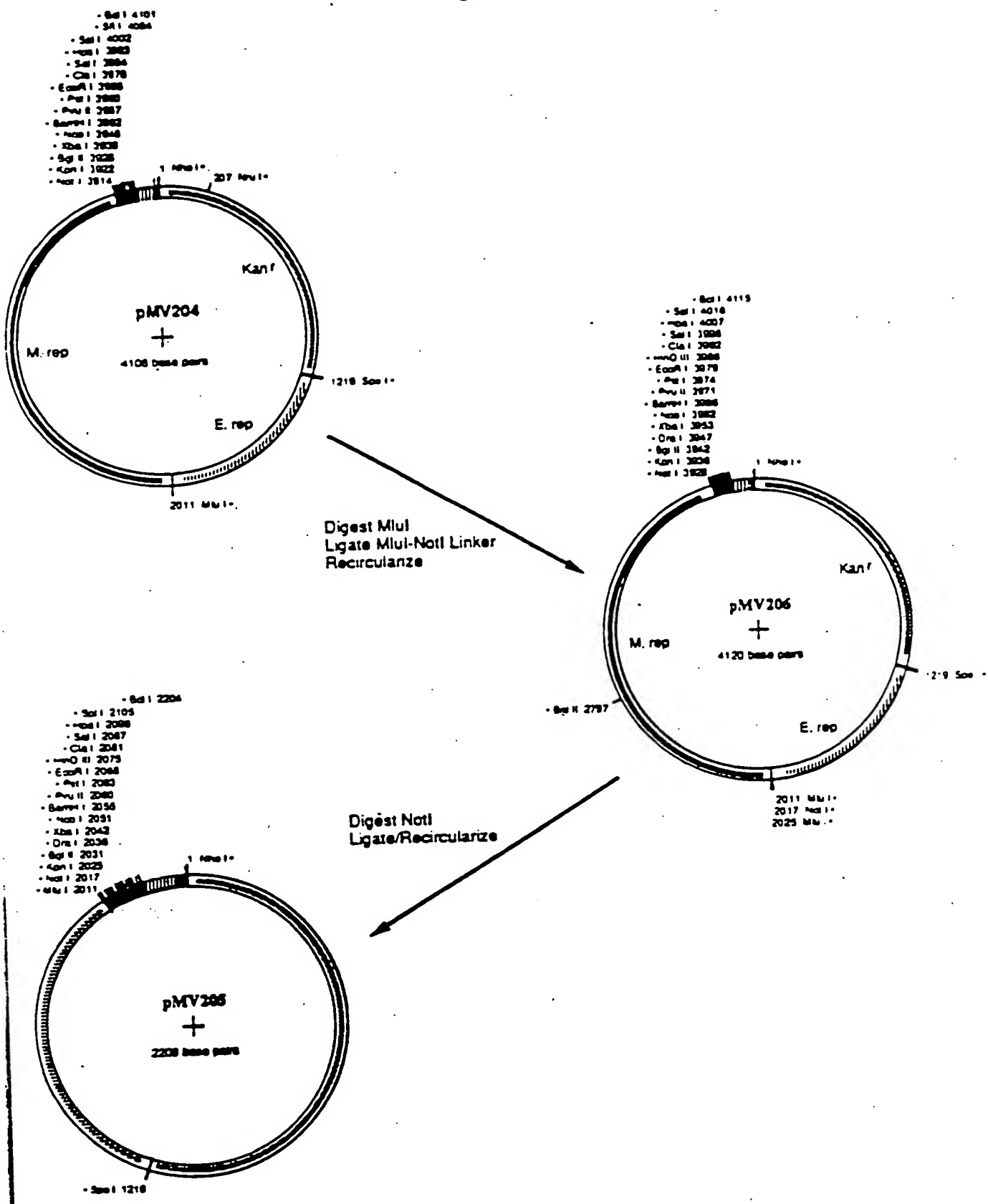

pMV204
4108 base pairs
Kan r
M. rep
E. rep
1 Nhe I
207 Nru I
1218 Sca I
2011 Mlu I
Digest Mlu I
Ligate Mlu I-Not I Linker
Recircularize
pMV206
4120 base pairs
Kan r
M. rep
E. rep
2011 Mlu I
2017 Not I
2025 Mlu I
Digest Not I
Ligate/Recircularize
pMV205
2208 base pairs
1 Nhe I
2011 Mlu I
2017 Not I
2025 Mlu I
1218 Sca I

Figure 30

pMV206

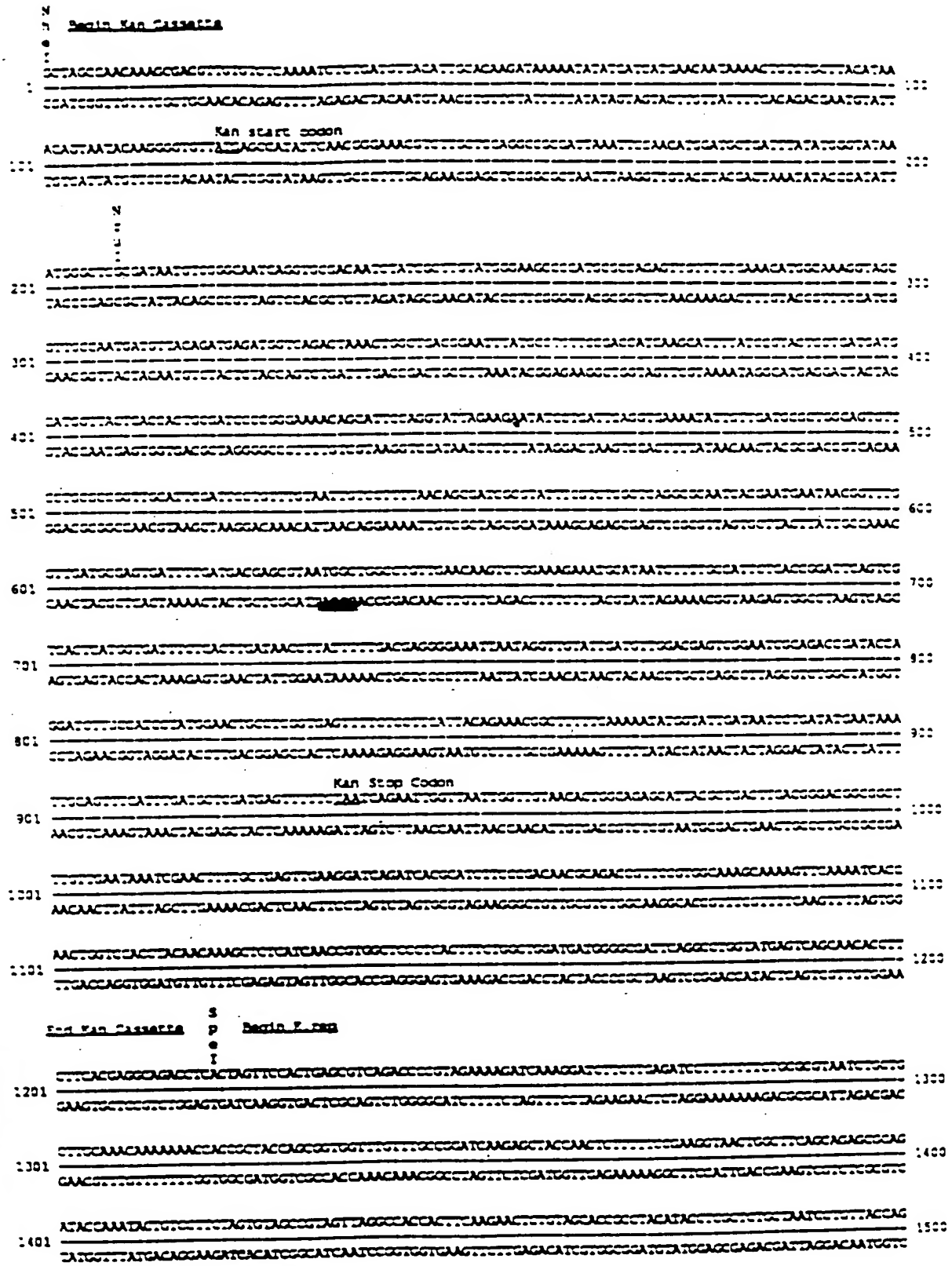
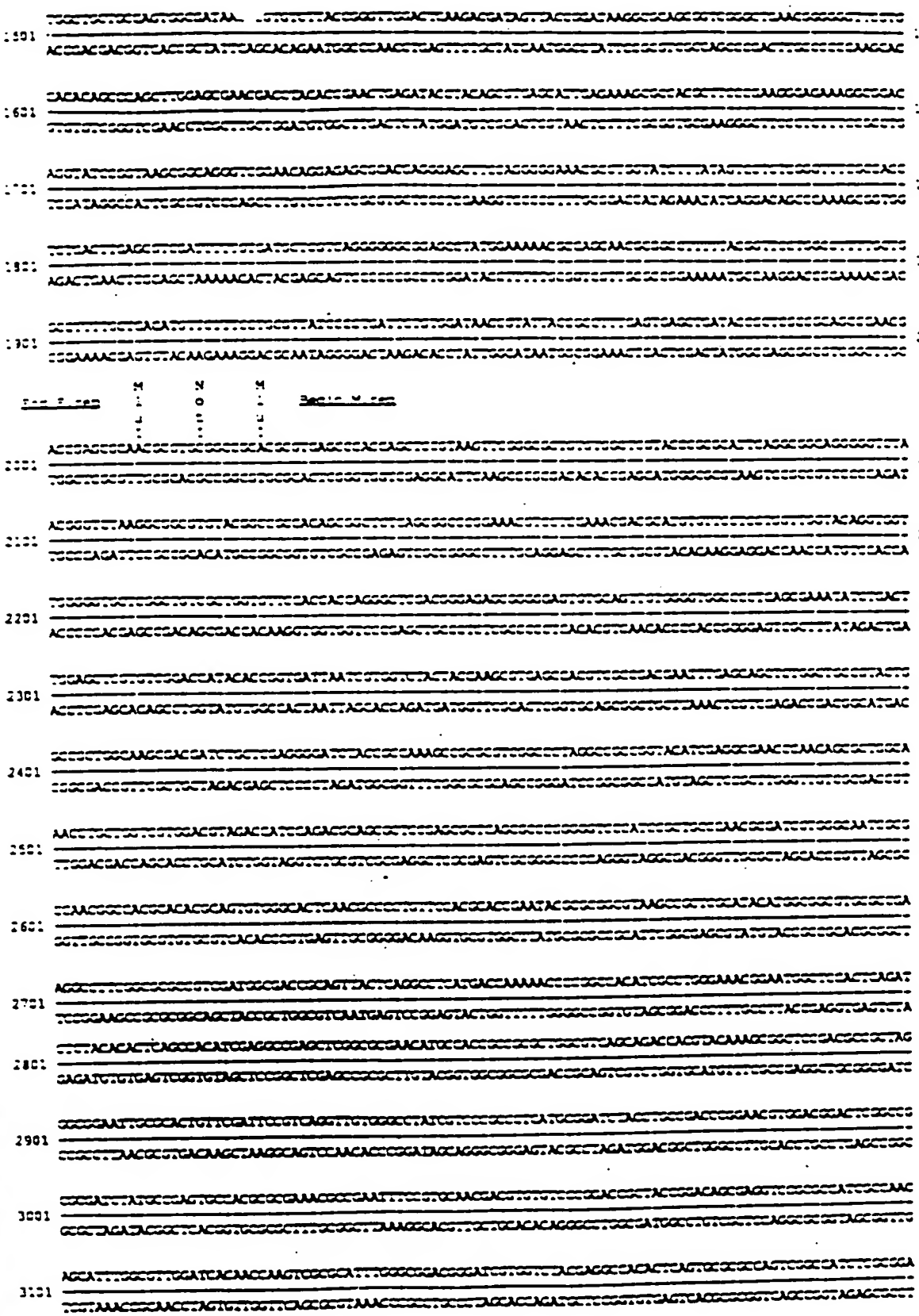
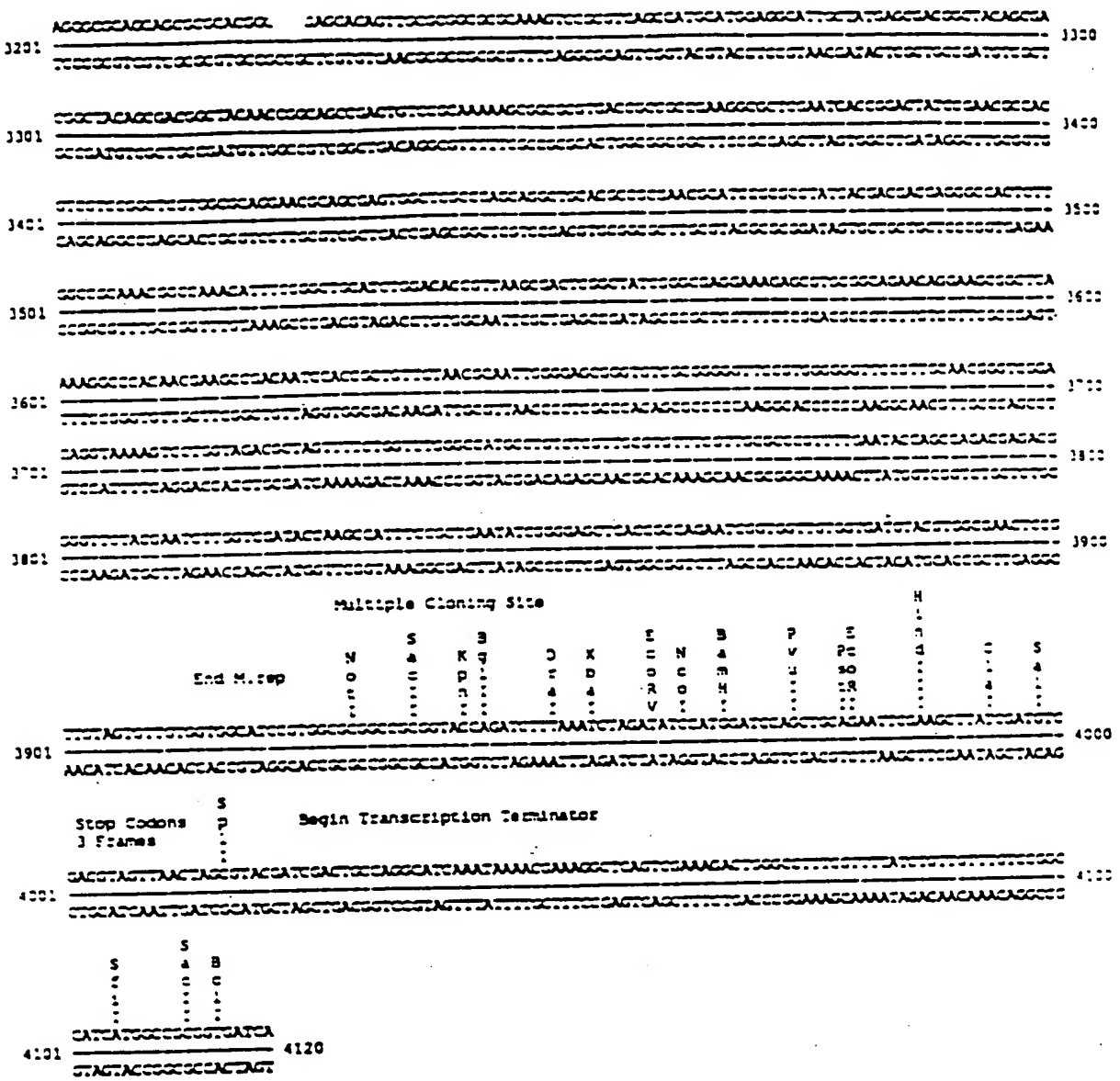
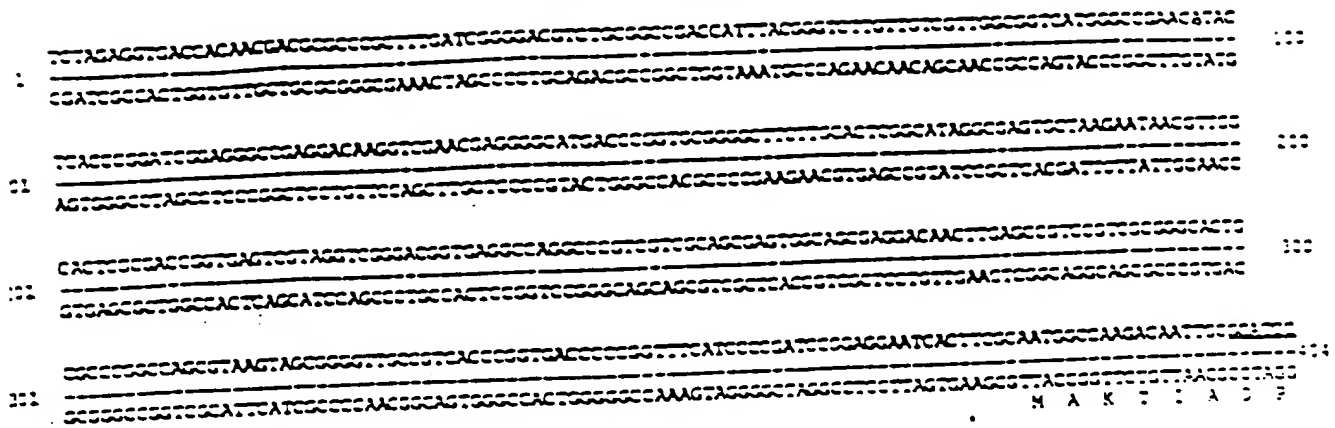


Figure 31




Multiple Cloning Site
End M.rep
Stop Codons 3 Frames
Begin Transcription Terminator

HSP61 Cassette



HSP71 Cassette

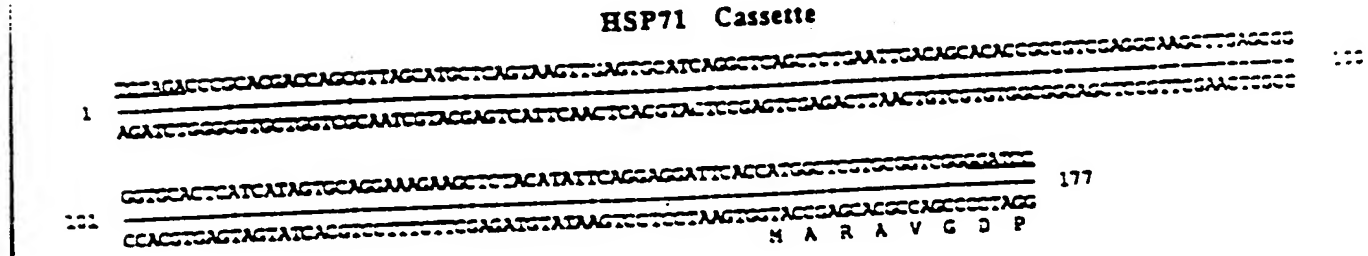
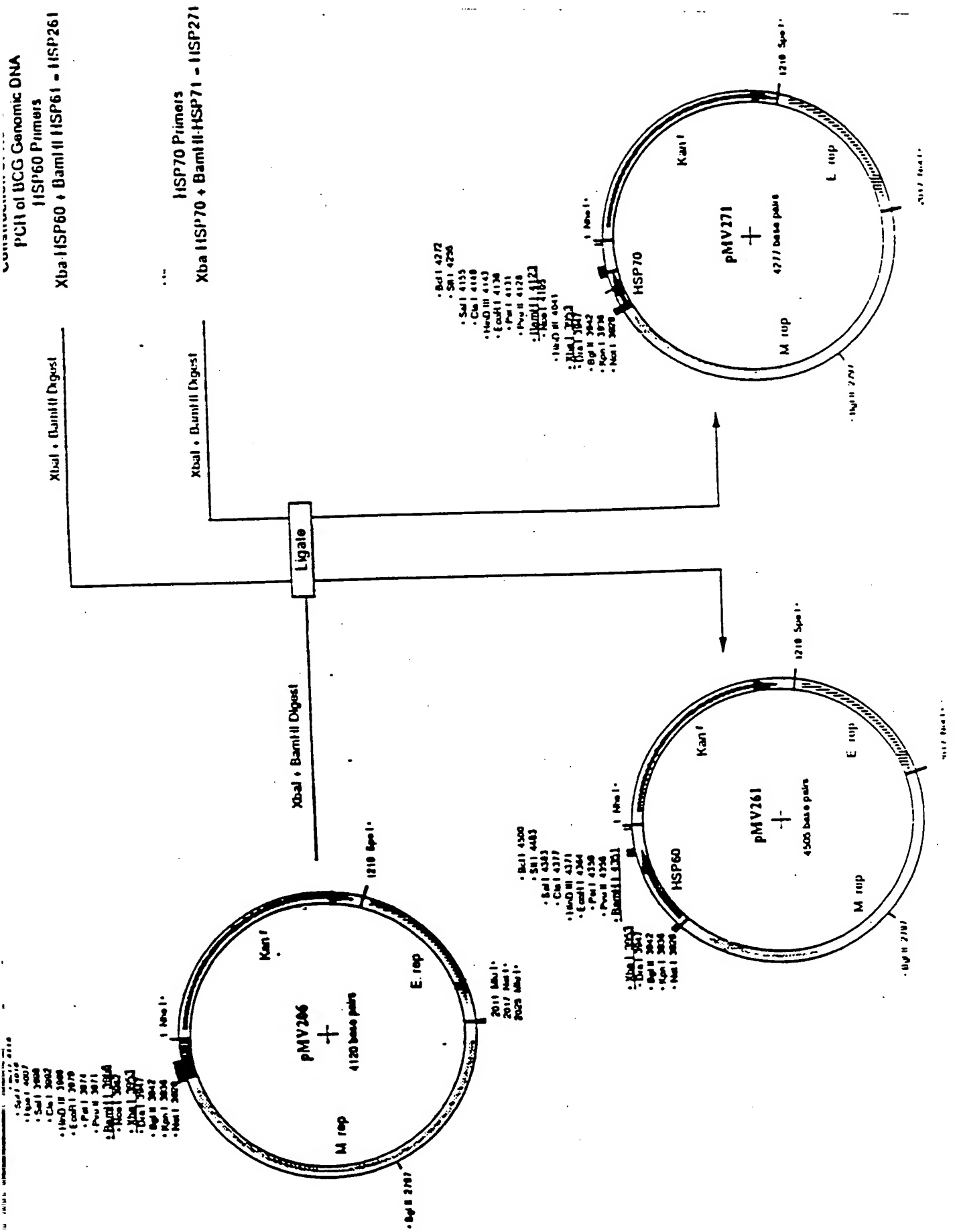


Figure 32


PCR of BCG Genomic DNA
HSP60 Primers
Xba HSP60 + BamHI HSP61 - HSP261
HSP70 Primers
Xba HSP70 + BamHI-HSP71 - HSP271
XbaI + BamHI Digest
XbaI + BamHI Digest
XbaI + BamHI Digest
Ligate
pMV271
4777 base pairs
pMV261
4505 base pairs
pMV266
4120 base pairs
HSP70
HSP60
Kan r
E. rep
M rep
1210 SpeI
1 NheI

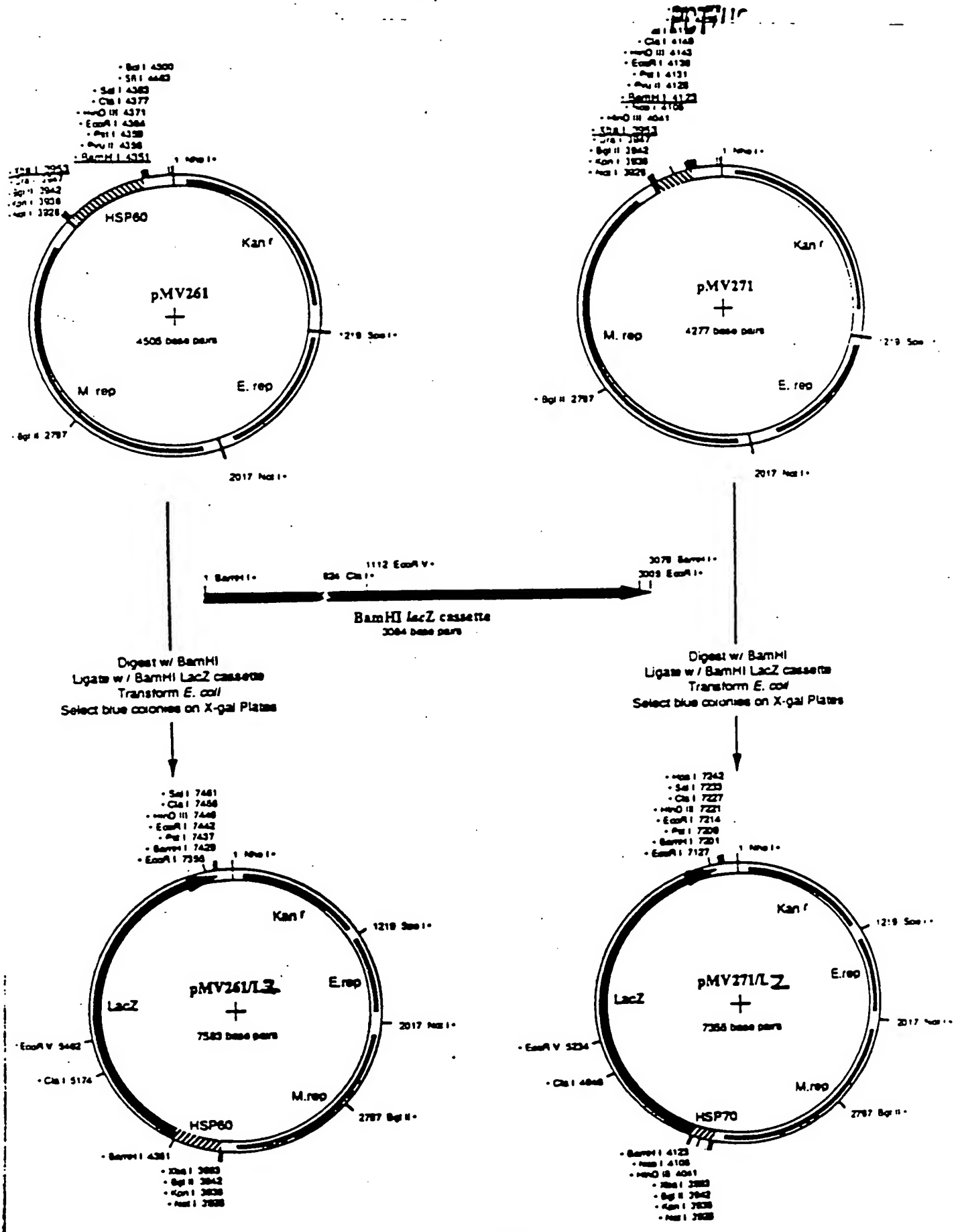

HSP60
Kan r
pMV261
4505 base pairs
M rep
E. rep
2017 Not I
Kan r
pMV271
4277 base pairs
M. rep
E. rep
2017 Not I
1112 EcoR V
1 BamH I
826 Cla I
3078 BamH I
3069 EcoR I
BamHI lacZ cassette
3084 base pairs
Digest w/ BamHI
Ligate w / BamHI LacZ cassette
Transform E. coli
Select blue colonies on X-gal Plates
Digest w/ BamHI
Ligate w / BamHI LacZ cassette
Transform E. coli
Select blue colonies on X-gal Plates
Kan r
pMV261/LZ
7583 base pairs
LacZ
E.rep
M.rep
HSP60
2017 Not I
2787 Bgl II
Kan r
pMV271/LZ
7356 base pairs
LacZ
E.rep
M.rep
HSP70
2017 Not I
2787 Bgl II

Figure 34

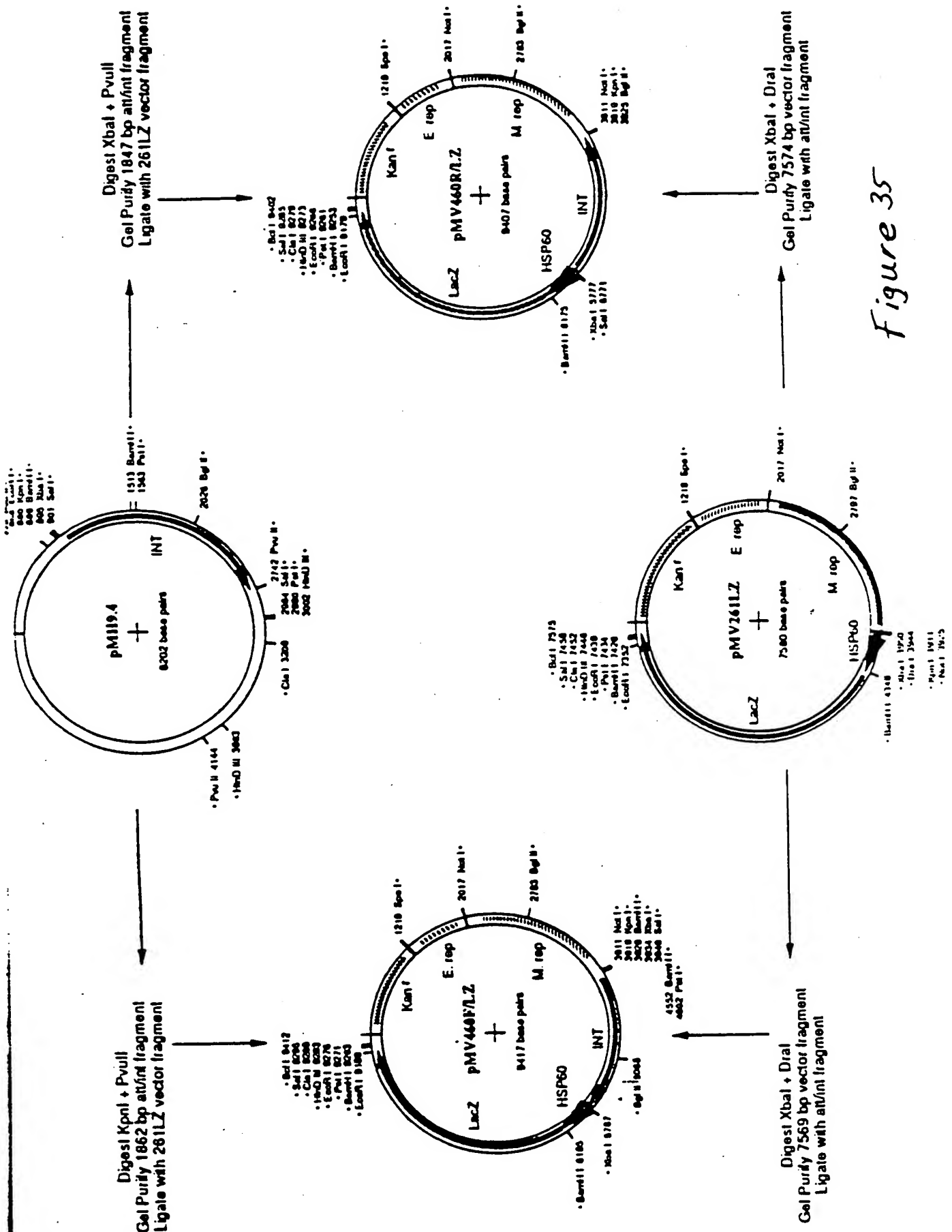
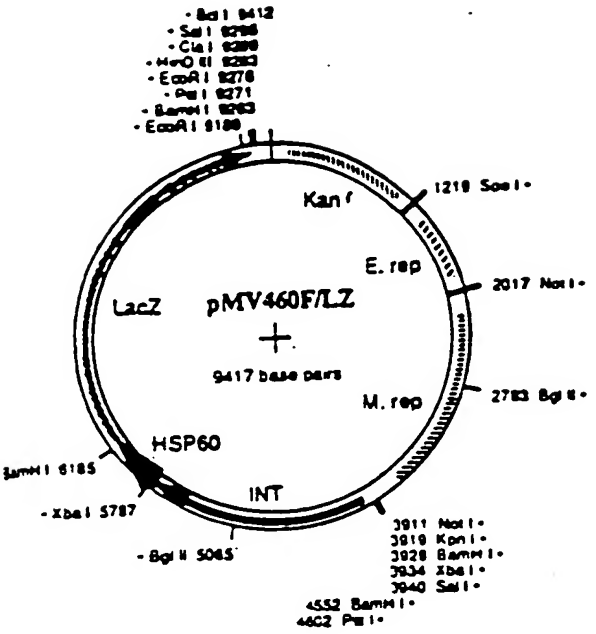
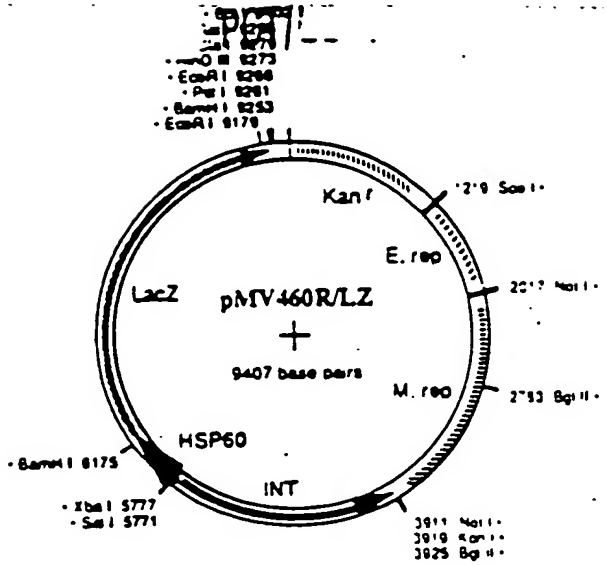

Digest Xbal + PvuII
Gel Purify 1847 bp att/int fragment
Ligate with 261LZ vector fragment
pMV460R/LZ
9407 base pairs
Kan r
E rep
M rep
INT
HSP60
Lac Z
Digest Xbal + DraI
Gel Purify 7574 bp vector fragment
Ligate with att/int fragment
pM119.4
8202 base pairs
Digest KpnI + PvuII
Gel Purify 1862 bp att/int fragment
Ligate with 261LZ vector fragment
pMV440R/LZ
9417 base pairs
Digest Xbal + DraI
Gel Purify 7569 bp vector fragment
Ligate with att/int fragment
pMV261LZ
7580 base pairs

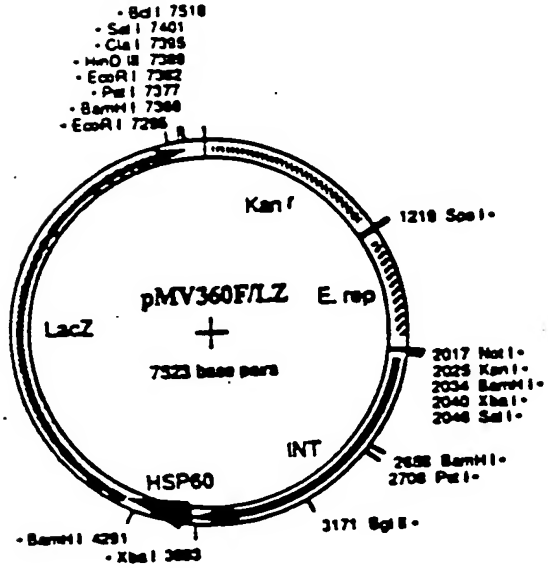
Figure 35





Digest with NotI
Gel Purify 7523 bp fragment
Ligate/Recircularize

Digest with NotI
Gel Purify 7513 bp fragment
Ligate/Recircularize



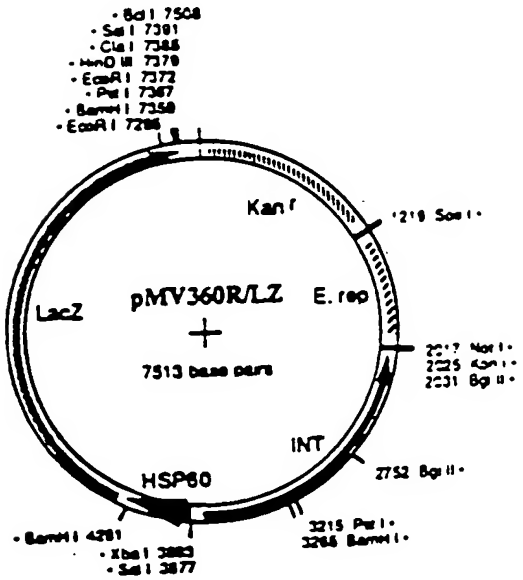


Figure 36

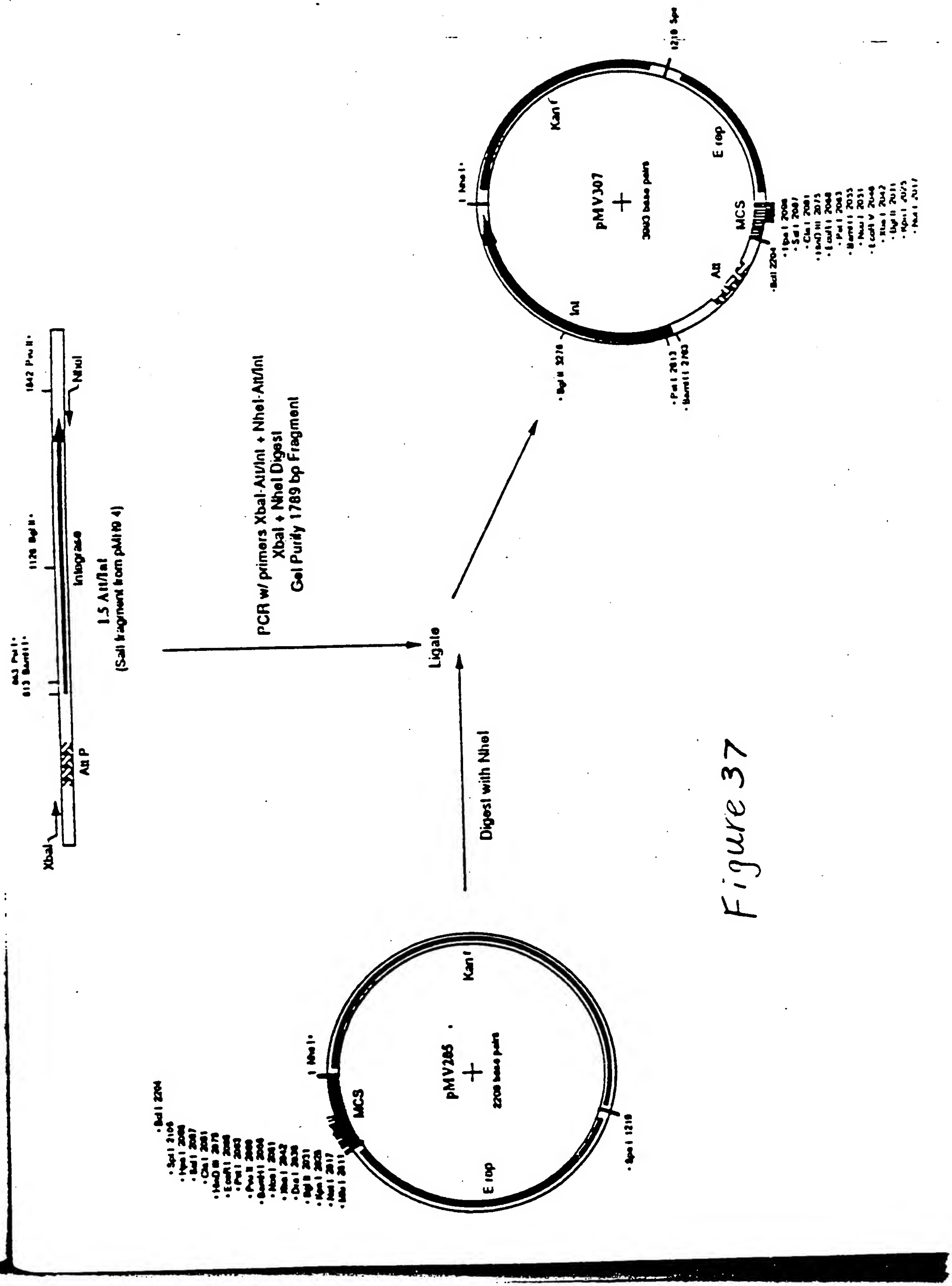

Att P
XbaI
NheI
Integrase
1.5 Att/Int
PCR w/ primers XbaI-Att/Int + NheI-Att/Int
XbaI + NheI Digest
Gel Purify 1789 bp Fragment
Ligate
Digest with NheI
pMV307
Kan r
MCS
Att
Int
pMV285
MCS
Kan r

Figure 37

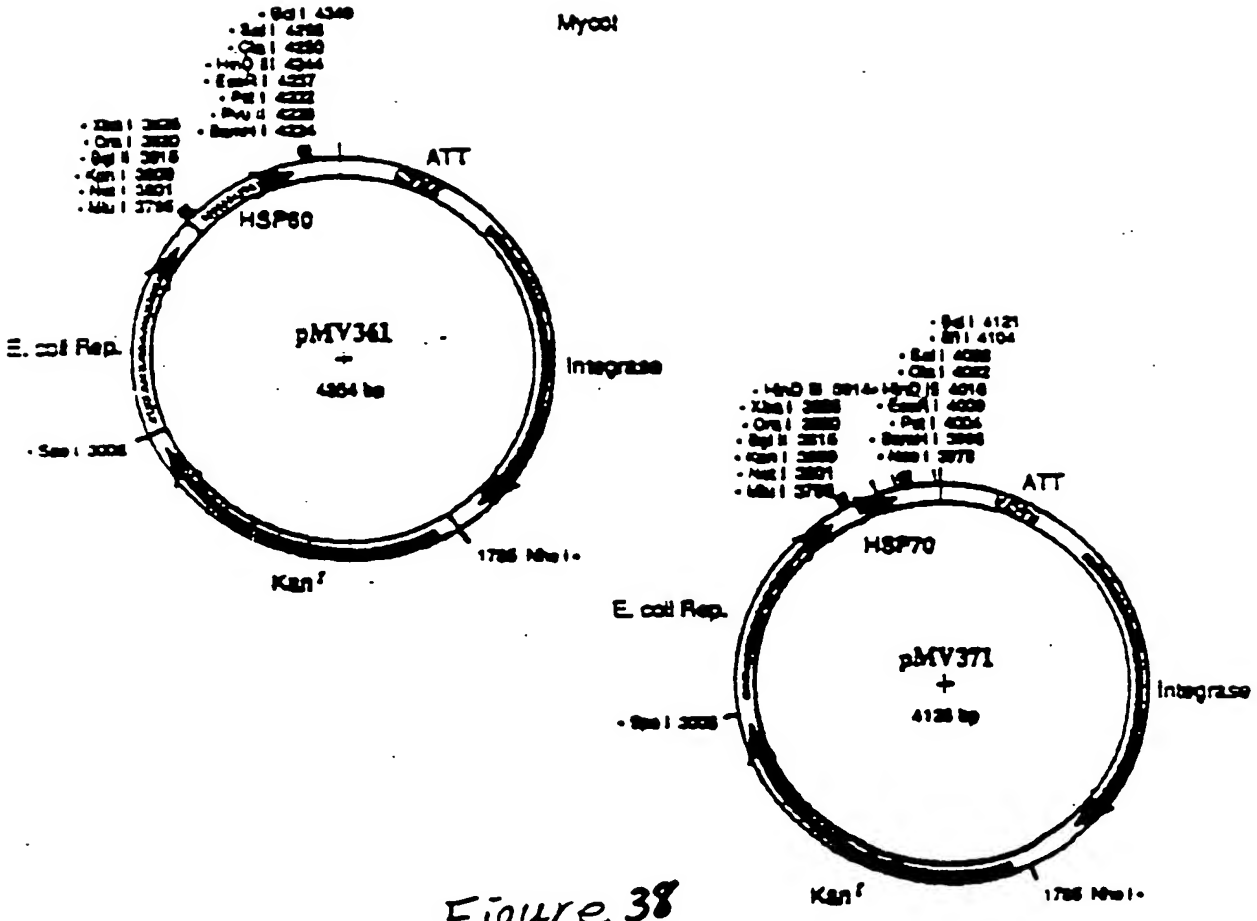

Mycot
ATT
HSP60
pMV361
+
4364 bp
E. coli Rep.
Integrase
Kan^r
1786 Nhe I
ATT
HSP70
pMV371
+
4126 bp
E. coli Rep.
Integrase
Kan^r
1786 Nhe I

Figure 38

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04833

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/00, 1/21, 15/11; A61K 39/00
U.S.Cl: 536/27; 435/69.1, 252.3; 424/92

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	536/27; 435/172.3, 252.3, 69.1; 424/92

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Databases: Dialog (files 5, 155, 350, 351, 399), US PTO Automated Patent System (File US Pat., 1975-1991)
search terms: mycobacter ? bacteriophage, integrat ?

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	WO, A, 90/00594 (Bloom et al) 25 January 1990, see pages 37-39, 64-70 and Figs. 6 7 .	1,2,8-11 3-7
X Y	WO, A, 88/06626 (Bloom et al) 07 September 1988, see entire document.	1, 2, 8-11 3-7
X Y	Proceedings of the National Academy of Sciences, volume 85, issued September 1988, Snapper et al., "Lysogeny and transformation in mycobacteria: Stable expression of foreign genes", pages 6987-6991, see the entire document.	1, 2, 8-11 3-7

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 October 1991	07 NOV 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Nancy Treptow

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	Reviews of Infectous Diseases volume 11, Supp. 2, issued March-April 1989, Jacobs, Jr. et al., Myco bacteriophage vector systems", pages 5404-5410, see entire document.	1, 2, 8-11 3-7
Y	Molecular and General Genetics, volume 195, issued May 1984, Pierson III et al., "Cloning of the integration and attachment regions of bacteriophage P4", pages 44-51, see the entire document.	7
Y	Molecular and General Genetics, volume 192, issued April 1983, Ljungquist et al., "Properties and products of the cloned int gene of bacteriophage P2," pages 87-94, see the entire document.	7
Y	Journal of Bacteriology, volume 170, No. 12, issued December 1988, Li et al., "Site-specific Integration and expression of a developmental promoter in Myxococcus Xanthus," pages 5552-5556, see entire document.	7
Y	Journal of Bacteriology, volume 171, No. 3, issued March 1989, Astumian et al., "Site-specific recombination between cloned attP and attB sites from the Haemophilus influenzae bacteriophage HP 1 propagated in recombination-deficient Escherichia coli" pages 1747-1750, see the entire document.	7

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